Molecular Characterization of Oligopeptidase B and Paraflagellar Rod (1 and 2) genes of *Trypanosoma evansi* Isolated from Camel

ऊँट से पृथक ट्रीपैनोसोमा इवान्सी के ***ओलीगोपेपटीडेज बी*** और पाराफ्लाजेलर रॉड (1 और 2) जीन के आन्विक अभिलक्षण

SANJAY KUMAR

M.V.Sc

THESIS

DOCTOR OF PHILOSOPHY

(Veterinary Parasitology)

।।पशुधनं नित्यं सर्वलोकोपकारकम्।।

2013

Department of Veterinary Parasitology,

College of Veterinary and Animal Science,

Rajasthan University of Veterinary and Animal Sciences, Bikaner – 334001 (Rajasthan)

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THESIS

Submitted to the
Rajasthan University of Veterinary and Animal Sciences, Bikaner
In partial fulfillment of the requirements for
the degree of

DOCTOR OF PHILOSOPHY

(Veterinary Parasitology)

BY

SANJAY KUMAR

M.V.Sc

2013

Rajasthan University of Veterinary and Animal Sciences, Bikaner
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CERTIFICATE - I

Date: ……..

This is to certify that **SANJAY KUMAR** has successfully completed the **PRELIMINARY EXAMINATION** held on 30-07-2011 as required under the regulations for the degree of **DOCTOR OF PHILOSOPHY.**

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CERTIFICATE - II

Date: ……..

This is to certify that this thesis entitled **"Molecular Characterization of Oligopeptidase B and Paraflagellar Rod (1 and 2) genes of *Trypanosoma evansi* Isolated from Camel"** submitted for the degree of **DOCTOR OF PHILOSOPHY** in the subject of **Veterinary Parasitology** of the Rajasthan University of Veterinary and animal Sciences, Bikaner, embodies bona-fide research work carried out by **Sanjay Kumar,** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during this work have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on 22/12/2012.

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CERTIFICATE - III

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This is to certify that the thesis entitled **"Molecular Characterization of Oligopeptidase B and Paraflagellar Rod (1 and 2) genes of *Trypanosoma evansi* Isolated from Camel"** submitted by **Sanjay Kumar**, to Rajasthan University of Veterinary and Animal Sciences, Bikaner, in partial fulfillment of requirements for the degree of **DOCTOR OF PHILOSOPHY** in the subject of Veterinary Parasitology after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination on his thesis has been found satisfactory, we therefore recommend that the thesis be approved.

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CERTIFICATE - IV

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This is to certify that **Sanjay Kumar**, of the **Department of Veterinary Parasitology**, College of Veterinary and Animal Science, Bikaner has made all the corrections/modifications in the thesis, entitled **"Molecular Characterization of Oligopeptidase B and Paraflagellar Rod (1 and 2) genes of *Trypanosoma evansi* Isolated from Camel"** which were suggested by the external examiner and advisory committee in the oral examination held on ..... The final copies of thesis duly bound and corrected were submitted on..... are enclosed here with for approval.

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
cDNA	Complementary Deoxy ribonucleic acid
D.W.	Distilled water
DEPC	Di ethyl pyro carbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
IPTG	Isopropyl – β- D- thiogalactoside
LB	Luria- Bertani
LMP	Low Melting Point

mRNA	Messenger ribonucleic acid
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain Reaction
PK Buffer	Proteinase K Buffer
PK Enzyme	Proteinase K Enzyme
R.T.	Room temperature
RE	Restriction enzyme
RNA	Ribonucleic acid
RNase	Ribonuclease
RMSD	Root-mean-square Deviation
RPM	Revolution per minute
SDS	Sodium dodecyl sulphate
SOC	Super Optimal Broath with Catabolite Repression
TAE	Tris- Acetate EDTA
TE	Tris EDTA
UV	Ultra Violet
X- gal	5 -bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Units of Measurement

%	Percentage
µg	Microgram
µl	Microlitre
^{0}C	Degree Celcius
A	Absorbance
bp	Base pair
cm	Centimeter
Da	Dalton
g	Gram (s)
h	Hour (s)
IU	International unit
Kbp	Kilo base pair
KDa	Kilo Dalton
Kg	Kilogram
M	Molar
ma	Milli ampere
mg	Milli gram
min	Minute (s)
Min	Minute

ml	Milli litre
mm	Mili meter
mM	Milli molar
ng	Nano gram
OD	Optical density
pmol	Picomole
sec	Second (s)
U	Unit
V	Volt
V / cm	Volt per centimeter
V / V	Volume / volume
W / V	

Weight / volume

Molecular Characterization of Oligopeptidase B and Paraflagellar Rod (1 and 2) genes of *Trypanosoma evansi* Isolated from Camel

Ph.D. Thesis

Department of Veterinary Parasitology,
College of Veterinary and Animal Science,
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Submitted by : Sanjay Kumar
Major Advisor : Dr. G. S. Manohar

ABSTRACT

The present study was carried out to isolate the Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *Trypanosoma evansi* using PCR/RT-PCR, clone the amplicons in a suitable plasmid vector and then characterization of above genes through sequencing. For this investigation, morphologically suspected *Trypanosoma evansi* infected camel was confirmed

by examination of Giemsa stained blood smear of camel blood. After confirming infection, the *T. evansi* collected from camel blood were propagated in Swiss albino mice and the blood of mice was collected from heart region after dissecting the mice which had massive infection. DEAE cellulose chromatography was done for purification of trypanosomes from blood of mice. DNA extraction was done from collected pellets of *Trypanosoma evansi* using the phenol-chloroform extraction followed by ethanol precipitation. Extraction of total RNA was done by Trizol reagent as conventional method as well as using Promega SV Total RNA isolation kit following the manufacturer protocol. c-DNA synthesis and its purification was done from extracted total RNA using Clontec RT-PCR Kit.

The desired amplicons of *opdB*, *pfr1* and *pfr2* genes were then amplified by PCR using gene specific primers (amplicons of *pfr1* and *pfr2* were also amplified by RT-PCR). Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide and identified on the basis of size of the *opdB*, *pfr1* and *pfr2* genes. The amplicons of expected size were purified from the 1% low melting agarose gel employing illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragment of interest was then ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid could be identified on the basis of blue/white colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinants was done by Restriction Enzyme digestion of plasmid DNAs using *Eco*RI and found that the release of DNA fragments around 2092 bp for *opdB*, 1769 bp for *pfr1* and 1767 bp for *pfr2* gene. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers. After confirmation of clones of *opdB*, *pfr1* and *pfr2* genes the plasmid DNAs were sequenced and coding sequences of *opdB*, *pfr1* and *pfr2* genes according to the results obtained were of 2092 bp, 1769 bp and 1767 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology of *opdB*, *pfr1* and *pfr2* gene is based on the Neighbor-Joining

method and maximum parsimony with 100% bootstrap values. Multiple sequence alignment of obtained protein sequences of *opdB*, *pfr1* and *pfr2* genes was performed with Clustal W (Clustal 2.1) at EBI. Identified *opdB*, *pfr1* and *pfr2* gene sequences showed a close homology with other *Trypanosoma* and *Leishmania spp*. gene sequences. 3D structure model of obtained opdB, pfr1 and pfr2 proteins have been determined by using homology modeling protocol. The final stable structure of opdB have 9 sheets, 4 beta alphabeta unit, 23 beta hairpins, 11 beta bulges, 39 strands, 22 helices, 20 helix-helix interfaces, 72 beta turns and 6 gamma turns. The stable structure of pfr1 have 4 sheets, 1 beta-alpha-beta unit, 2 beta hairpins, 2 beta bulges, 9 strands, 35 helices, 57 helix-helix interfaces, 105 beta turns and 16 gamma turns and pfr2 have 4 helices, 4 helix-helix interact, 3 beta turns and 2 gamma turns.

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Place: Bikaner

Date: *Sanjay Kumar*

APPENDIX – I

1. Agarose Gel Electrophoresis buffer

1.1. TAE buffer (50x)

Stock solution:

Tris base	121	gm

Glacial acetic acid	28.55 gm
0.5 M EDTA acid (pH 8.0)	50 ml
Water upto	500 ml

Working concentration of TAE buffer (1x)

TAE buffer	10 ml
Water	490 ml

1.2. TE (Tris/EDTA) Buffer

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM

Make from 1M stock of Tris-HCl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

Working solution:

1M Tris-HCl (pH 7.5)	1.0 ml
500mM EDTA (pH 8.0)	0.2 ml
Water to	100 ml

2. Phosphate Buffered Saline buffer (1X), pH 7.4

NaCl	8.0 gm
$Na_2HPO_4.2H_2O$	1.44 gm
KH_2PO_4	0.24 gm
KCl	0.20 gm
Distilled water to make	1000 ml

3. Trypanosome separation buffer (PSG buffer, pH 8.0)

3.1. Solution A

Na_2HPO_4 (anhydrous)	8.000 gm
$NaH_2PO_4.2H_2O$	0.780 gm
NaCl	4.250 gm
Distilled water to make	1000 ml

3.2. Solution B

Glucose solution dextrose	10 gm
Distilled water to make	400 ml

Just before use 6 parts of solution A was mixed with 4 part of solution B

4. Proteinase K buffer

Tris base (ph 8)	100mM
EDTA	10mM
NaCl	50mM
SDS	2%
β Mercapto ethanol	20mM

APPENDIX – II

1. Giemsa stain

Giemsa stain	1 ml
Distilled water	9 ml

Slides were stained for 45 minutes.

2. 6X loading dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	50%
EDTA	2 mM

3. Ethidium bromide solution (10 mg/ ml)

Ethidium bromide	0.2gm
Sterile Water	20 ml

4. Agarose (0.8%)

Agarose	800 mg
TAE buffer	100 ml

5. Agarose (1.2%)

Agarose	1.2 gm
TAE buffer	100 ml

APPENDIX – III

1. Phenol: Chloroform: Iso amyl alcohol; 25:24:1

Phenol	250 ml
Chloroform	240 ml
Iso amyl alcohol	100 ml

2. Alcohol (70%)

Alcohol	70 ml
Water	30 ml

3. Luria Bertani (LB) Medium (500 ml)

Tryptone	1.0% (5 gm)
Yeast extract	0.5% (2.5 gm)
NaCl	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

For LB plates, add 1.5% (7.5 gm) agar to the LB broth and autoclave.

4. Luria Bertani (LB) Agar (500 ml)

Tryptone	1.0% (5 gm)
Agar	1.5% (7.5 gm)
Yeast extract	0.5% (2.5 gm)
NaCl	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

5. LB plates with ampicillin/ IPTG/ X- Gal

LB plates with ampicillin were made by adding ampicillin to a final concentration of 50µg/ml after cooling of LB agar to 50^0C. Then 100 µl of 100mM IPTG and 20 µl of 20mg/ml X- Gal was spreaded over the surface of the LB- ampicillin plate and allowed to absorb for 30 minutes at 37ºC prior to use.

6. SOC medium (100ml)

Tryptone	2 gm
Yeast extract	0.5 gm
NaCl (1M)	1 ml
KCl (1M)	0.25 ml
Mg+2 stock (2M)	1ml
Glucose (2M)	1ml

2M Mg^{+2} stock (100ml)

$MgCl_2 . 6H_2O$	20.33 gm
$MgSO_4 . 7H_2O$	24.65 gm

7. X-GAL solution

X-GAL	20mg/ml

Dissolved in 100% N,N dimethyl formamide.

20 µl X-GAL was used for 25-30ml LB agar medium in LB plate.

8. IPTG solution

IPTG	100mM

Dissolved in distilled water.

100µl IPTG was used for 25-30ml LB agar medium in LB plate.

9. Ampicillin solution

Ampicillin 50µg/ml

Dissolved in distilled water as 10mg/ml stock.

To make 25ml of LB broth medium containing 50µg/ml Ampicillin, 125 µl of 10mg/ml ampicillin stock was used.

For 500 ml LB agar 2.5 ml of 10 mg/ml ampicillin stock was added, ensure that LB agar cooled to 50°C before adding ampicillin.

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5. DISCUSSION

Trypanosomes are unicellular parasites with a single flagellum, which belong to the order kinetoplastida. Kinetoplastid protozoa, including Trypanosoma and other genera, are ancient eukaryotic organisms that infect wide range of hosts, ranging from plants to invertebrates to mammals. Trypanosomes are obligate parasite of vertebrates having digenetic life cycle, alternating between vertebrate and invertebrate host. In most domestic and many wild animals *Trypanosoma evansi* is highly pathogenic with

clinical signs depending on strain pathogenicity, host species, general stresses on the host and local epidemiological conditions. The most common clinical signs include fever, anaemia, oedema, emaciation, abortion and enlargement of lymph nodes, liver and spleen and occasional neurological signs. As discussed earlier they affect various domesticated animals including camels, horses, cattle, buffaloes, mules, sheep, goat, dog, cats and pigs. Camels usually develop acute disease, though they may survive to develop chronic infection or even undergo spontaneous recovery. Therapeutic strategies against *Trypanosoma evansi* infection have their limitations with the continuous emergence of drug resistance. Besides, vaccines are also lacking against this organism due to its antigenic variations. The study on “Molecular characterization of Oligopeptidase B and Paraflagellar rod (1 and 2) genes of *Trypanosoma evansi* isolated from camel” which was undertaken in present work could be helpful for understanding the pathogenesis of trypanosomosis, development of more effective drugs and evolution of vaccines.

Oligopeptidase B is an important virulence factor and therapeutic target in trypanosome infections. It is a parasite peptidase that inactivates atrial natriuretic factor in the bloodstream of infected hosts. According to reports of Caler *et al*. (1998) deletion of the gene encoding oligopeptidase B in *Trypanosoma evansi* results in a marked defect in host cell invasion and in the establishment of infections in mice. Studies of Troeberg *et al.* (1996) indicated that the *T. brucei* oligopeptidase, called OP-Tb, may play an important direct role in the pathogenesis of African trypanosomiasis through the degradation of regulatory peptide hormones in the blood of infected hosts. During infection, OP-Tb is released into the host bloodstream, where it is insensitive to serum protease inhibitors. In the South American trypanosome, *Trypanosoma cruzi,* the proteolytic activity of opdB is required for calcium-signaling, which regulates trypanosome invasion of the host cell. OpdB achieves this by generating an active signaling ligand that interacts at the host cell surface, mobilizing intracellular calcium and promoting the trypanosome invasion (Burleigh and Andrews, 1995;

Burleigh *et al*., 1997; Caler *et al*., 1998). Targeted deletion of the *opdB* gene in *T. cruzi* trypomastigotes significantly decreased the parasite virulence to mammalian host cells giving reduced parasitaemia in mice (Caler *et al*., 1998).

The paraflagellar rod (PFR) is one of several unique attributes characterizing the biology of kinetoplastid protozoa and has served as a focus for speculation since its first identification by Keith Vickerman in 1962 (Vickerman, 1962). PFR1 and PFR2 are highly abundant proteins and considered as the classic defining components of the paraflagellar rod in Trypanosomes. These PFR can assume two different conformations, resulting in two distinct bands of apparent molecular masses 73 and 69 kD in SDS-gel electrophoresis (Schlaeppi *et al*.; 1989). Both the PFR proteins are intact to flagellum and hence play major role in cellular motility. They are major structural components of the paraflagellar rod and identified as highly pathogenic genes. These *pfr1* and *pfr2* genes are logical targets for therapeutic intervention in the infection process and also suggested as attractive vaccine agent for prevention or control of Trypanosomes infection. Due to its unique nature, the PFR has been raised by several authors as a target for the fight against parasites such as *T. cruzi*, African trypanosomes and *Leishmania* spp, of critical medical and/or socio-economical importance (Hunger-Glaser and Seebeck 1997; Wrightsman *et al*. 1995). Injection of mice with *T. cruzi* PFR proteins confers protection against an otherwise lethal challenge of trypomastigotes (Wrightsman *et al*. 1995). This protection is cellular mediated rather than humoral (Miller *et al*. 1997) and might be exploited in the development of a vaccine against this parasite.

During the present study initially, *T. evansi* were isolated from the naturally infected camel at NRCC, Bikaner. Parasites were propagated in laboratory animals (mice) after injecting intra-peritoneally and pure isolates were obtained by column chromatography of infected blood collected from heart of mice. Genomic DNA was synthesized from these pure isolates of *T. evansi.* cDNA synthesis was also carried out through total RNA extracted from these isolates. Following this, the desired genes, *opdB*, *pfr1* and *pfr2* were amplified employing PCR. *pfr1* and *pfr2* genes were also amplified by using RT-PCR. The purified PCR

products (amplicons) thus obtained were cloned into suitable bacterial vector (pGEM-T Easy). The genes were sequenced from purified plasmid of the obtained clones.

The sequences of desired genes were compared with other related species gene sequences. After analysis, the gene sequences showed a wide range of homology within the organisms of Trypanosomatidae family.

Obtained Oligopeptidase B gene sequence showed 99.6% homology towards *T. brucei*, GenBank Accession No. AF078916 and 99.0% similarities with *T.brucei.* GenBank Accession No. XM824253. A 100 percent homology was found between obtained opdB sequence and *T. evansi*, GenBank Accession No. AY546084. Lower homology was documented between the obtained *opdB* gene sequence and *T. cruzi*, GenBank Accession No. XM804874 and *T. cruzi*, GenBank Accession No. U69897. The comparison with *Leishmania amazonensis* (GenBank Accession No. EF392367), *Leishmania donovani* (GenBank Accession No. GQ491028), *Leishmania infantum* (GenBank Accession No. XM001463502) and *Leishmania major* (GenBank Accession No. AF109875) a member belonging to Trypanosomatidae family showed only 77.6%, 76.2%, 75.9% and 76.2% homology, respectively.

Based on the above homology, *Trypanosoma cruzi,* GenBank Accession No. XM804874 and *T. cruzi,* GenBank Accession No. U69897 in the phylogenetic tree are placed as two sub cluster of one mega cluster. *Leishmania major* (GenBank Accession No. AF109875), *Leishmania amazonensis* (GenBank Accession No. EF392367), *Leishmania donovani* (GenBank Accession No. GQ491028) and *Leishmania infantum* (GenBank Accession No. XM001463502) as four sub cluster of one mega cluster; the other mega cluster comprising of rest of the species. The earlier reports of *T. evansi* by Morty *et al.* (2005) also showed 99.6% homology with *T. brucei*, GenBank Accession No. AF078916 and 99.0% homology with *T. brucei*, GenBank Accession No. XM824253. 75.0% to 77.6% sequence similarities was observed between *T. evansi opdB* gene and other species documented in this study.

The *opdB* gene isolated from *T. evansi* by Morty *et al.* (2005) contained an open reading frame of 2148 bp encoding a polypeptide of 715 amino acids with a predicted molecular mass of 80.664 kDa. This is the first protease- encoding gene cloned and sequenced from *T. evansi*. The *T. evansi opdB* gene was present as a single copy per haploid genome as judged by Southern blot of endonuclease- restricted *T. evansi* genomic DNA probed with the full length *opdB* coding sequence. A complete open reading frame of oligopeptidase B from *Leishmania amazonensis* was amplified by Matos Guedes *et al.* (2007) with polymerase chain reaction using primers designed for the oligopeptidase B gene from *L. major*. The 2,196-bp fragment coded for a protein of 731 amino acids with a predicted molecular mass of 83.49 KDa. The encoded protein (La_OpB) shares a 90% identity with oligopeptidases of *L. major* and *L. infantum*, 84% with *L. braziliensis*, and ~67% identity with *Trypanosoma* peptidases.

In the present study, the paraflagellar rod 1 gene sequence showed 99.9% homology with *T. evansi*, GenBank Accession No. EU366960, 99.8% with *T. brucei*, GenBank Accession No. XM838928 and 99.4% with *T. brucei,* GenBank Accession No. Z25827. 100% sequence similarity was found between obtained *pfr1* gene sequence and *T. evansi*, GenBank Accession No. FJ968743. Slightly lower homology was documented between the obtained *pfr1* gene sequence and *T. cruzi*, GenBank Accession No. XM804737 and *T. cruzi*, GenBank Accession No. AF005195. The comperison with *Leishmania infantum,* GenBank Accession No. AY702344, *Leishmania major,* GenBank Accession No. XM003722211 and *Leishmania infantum,* GenBank Accession No. XM003392645 a member belonging to Trypanosomatidae family showed only 83.6%, 83.6%, and 83.9% homology, respectively.

According to neighbor-joining phylogenetic tree analysis of *pfr1* gene, *T. cruzi,* GenBank Accession No. XM804737 and *T. cruzi*, GenBank Accession No. AF005195 are placed as two sub cluster of one mega cluster. *Leishmania major,* GenBank Accession No. XM003722211, *Leishmania infantum,* GenBank Accession No. AY702344 and *Leishmania infantum,* GenBank Accession No. XM003392645 as three sub cluster of one mega cluster and the other mega cluster comprising of rest of the

species. Exactly same type of sequence similarities was observed between *T. evansi*, GenBank Accession No. FJ968743 and other documented species. The earlier reports of *T. evansi* by Abdille *et al.* (2008a) showed 99.9% homology with obtained *pfr1* sequence and sequence of *T. evansi*, GenBank Accession No. FJ968743 and very few differences in sequence similarities were observed between these sequences when compared with other documented species.

Sequence of Paraflagellar rod 2 gene obtained in present study (Accession No. JX020770) showed 99.9% homology towards *T. evansi,* GenBank Accession No. EU258755, *T. brucei,* GenBank Accession No. XM842234 and *T. brucei*, GenBank Accession No. X14819. 99.8% homology was found between obtained *pfr2* sequence and *T. evansi*, GenBank Accession No. GQ392136 and *T. evansi*, GenBank Accession No. FJ901341. The comparison with *T. brucei,* GenBank Accession No. L30155, *T. cruzi,* GenBank Accession No. M97548, *T. cruzi,* GenBank Accession No. FJ222461 and *T. cruzi,* GenBank Accession No. XM809076 a member belonging to Trypanosomatidae family showed 99.5%, 83.7%, 83.7%, and 83.8% homology, respectively.

Based on the above homology of various sequences of *pfr2* gene, *T. cruzi,* GenBank Accession No. XM809076, *T. cruzi,* GenBank Accession No. M97548 and *T. cruzi,* GenBank Accession No. FJ222461 in the phylogenetic tree are placed as three sub cluster of one mega cluster; the other mega cluster comprising of rest of the species. The earlier sequence report of *T. evansi pfr2* gene by Ghorui *et al.* (2009) also showed same type of homology as obtained *pfr2* sequence with other documented species sequences in this study. But *T. evansi pfr2* gene sequence reported by Abdille *et al.* (2008) from China showed 100% similarity with sequences of *T. brucei,* GenBank Accession No. XM842234 and *T. brucei,* GenBank Accession No. X14819 and as compared to obtained *pfr2* sequence, minor differences in sequence similarity was observed with other documented species sequences.

In open reading frame of nucleotide sequence of *pfr1* gene in *Trypanosoma evansi* of Izatnagar isolate from India (Maharana *et al*.; 2011a) revealed 99.8% homology with the China isolates and only one nucleotide change at 867 bp was detected. The nucleotide sequence of Izatnagar isolate also showed 99.8%, 82.1%, 79.9%, 72.9% homology with *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania infantum* and *Crithidia daenei*, respectively. The deduced amino acid sequence of *T. evansi pfr1* revealed 99.7% homology between Izatnagar and China isolate. The nucleotide sequence of *pfr2* gene of Izatnagar isolate from India (Maharana *et al*.; 2011b) showed 99.9% homology with the China isolates and also showed 99.9%, 82.4%, 75.3% and 74.8% sequence homology with the published sequence of *Trypanosoma brucei, T. cruzi, Leishmania infantum* and *Crithidia fasciculata*, respectively. The size of *pfr2* gene of *T. evansi* (Abdille *et al.;* 2008d) is consistent with that of *pfr2* genes from two other trypanosome species, *T. brucei* (GenBank Accession No. XM 842238) and *T. cruzi* (GenBank Accession No. XM 809076). The molecular weight of PFR2 protein of *T. evansi* observed by Abdille *et al.* (2008d) is about 69 kDa, which is consistent with the molecular weight of the PFR2 protein of *T. brucei.*

Trypanosomes have the capacity for antigenic variation, which is the basis of their ability to escape the host immune response and because of this, prospects for the development of a vaccine against trypanosomosis have been considered poor. Trypanosomes change their variant surface glycoprotein coat very quickly which is the main hurdle in vaccine development. Target of alternative invariant proteins like protozoan proteases and paraflagellar rod proteins may be helpful in vaccine evolution against trypanosomosis. An option for developing vaccines and chemotherapeutic agents against trypanosomosis is to target pathogenic factors released by the parasite during infection, namely an “anti-disease” approach (Authie *et al*., 2001). One such pathogenic factor is oligopeptidase B, a trypanosome peptidase that hydrolyses Arg/Lys containing peptides smaller than 30 amino acid residues and is suspected to be involved in the hormonal deregulation associated with the disease. In *T. evansi*, opd B has been

implicated in the inactivation of host atrial natriuretic factor (ANF), resulting in an increased blood volume, which is associated with lesions reported in the circulatory system of trypanosome infected hosts (Morty *et al*., 1999a & 2005a). Other important pathogenic factor in Trypanosomes infection is Paraflagellar rod proteins which is logical target for therapeutic intervention in the infection process and also suggested as attractive vaccine agent for prevention or control of Trypanosomes infection. This Paraflagellar rod proteins present in the kinetoplastid flagellum is a unique structure of trypanosoma flagellum due to presence of paracrystaline structure. PFR is vital for trypanosome motility (Bastin *et al*., 1998) and is unique among the kinetoplastids as their heteropolymers provide the building block of flagellum (Abdille *et al*., 2008d).

The principal finding of the present study was the identification of the *opdB*, *pfr1* and *pfr2* gene in *T. evansi* from camel by sequencing the recombinant plasmid pGEM-T Easy –*opdB*/*pfr1*/*pfr2* in both directions with forward and reverse primers. The present findings therefore suggest that the identified opdB gene showed a close homology with *T. brucei* (GenBank Accession No. XM824253 and GenBank Accession No. XM824253)), *T. evansi* (GenBank Accession No. AY546084) and *T. cruzi* (GenBank Accession No. XM804874 and GenBank Accession No. U69897). Identified *pfr1* gene also found in close homology with other Trypanosome sequences like *T. brucei* (GenBank Accession No. XM838928 and GenBank Accession No. Z25827), *T. evansi* (GenBank Accession No. FJ968743 and GenBank Accession No. EU366960) and *T. cruzi* (GenBank Accession No. XM804737 and Gene bank Accession No. AF005195). Similarly identified *pfr2* gene was quite similar to *T. brucei* (GenBank Accession No. XM842234, GenBank Accession No. X14819 and Gene bank Accession No. L30155), *T. evansi* (GenBank Accession No. GQ392136 and GenBank Accession No. FJ901341) and *T. cruzi* (GenBank Accession No. M97548 and GenBank Accession No. XM809076) sequences. The sequence identity of obtained *pfr1* and *pfr2* nucleotide sequences of *T. evansi* with other Trypanosomes species indicating that these *pfr1* and *pfr2* genes are highly conserved in the kinetoplastid species. As mentioned in

results amino acid sequences of obtained opdB, pfr1 and pfr2 proteins of *T. evansi* showed high level of homology with amino acid sequence of respective proteins of other trypanosomes species. A recent report described a similar case where amino acid sequence of the *T. evansi* beta-tubulin was found to be 100% and 99.8% identical to that of *T. equiperdum* and *T. brucei*, respectively (Li *et al*., 2007). It could therefore be hypothesized and suggested that vaccine with opdB, pfr1 and pfr2 proteins of trypanosomatidae parasite as the antigen could be effective against not only different strains within one trypanosome species but also against other species of the same genus.

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. Therefore, aforementioned experiments are attempted to characterize the genes of prime importance in *T. evansi* from Indian dromedaries. These genes would be the ideal vaccine and drug target in its own right for the control of trypanosomosis in India.

ऊँट से पृथक ट्रीपैनोसोमा इवान्सी के *ओलीगोपेपटीडेज बी* और पाराफ्लाजेलर रॉड (1 और 2) जीन के आन्विक अभिलक्षण

पीएच.डी. शोध ग्रंथ,

i'kq ijthoh foKku foHkkx]

i'kq fpfdRlk ,oa i'kq foKku egkfo|ky;]

jktLFkku i'kq fpfdRlk ,oa i'kq foKku fo'ofo|ky;]

chdkusj&334 001

शोधकर्ता- **संजय कुमार**

मुख्य उपादेष्टा- **डॉ. जी. एस. मनोहर**

अनुक्षेपण

वर्तमान अध्ययन में *ट्रीपैनोसोमा इवान्सी* का ***ओलीगोपप्टीडेज़ बी,*** पाराफ्लाजेलर रॉड 1 और पाराफ्लाजेलर रॉड 2 जीन को पी.सी.आर./आर.टी.-पीसीआर का उपयोग कर अलग किया गया, एक उपयुक्त प्लाज्मिड वेक्टर में एमप्लीकोंस को क्लोन किया गया और उसका बाद अनुक्रमण का माध्यम स उपरोक्त जीनों की विशेषता का अध्ययन किया गया। ऊंट खून का स्टेन्ड स्मीयर की परीक्षा का द्वारा आकृति का आधार पर संदिग्ध *ट्रीपैनोसोमा इवान्सी* संक्रमित ऊंट की पुष्टि की गई। संक्रमण की पुष्टि करना का बाद ऊंट रक्त स एकत्र *ट्रीपैनोसोमा इवान्सी* को स्विस अल्बिनो चूहों में विस्तारित किया गया और जब चूहों में भारी संक्रमण हो गया तब चूहों को विच्छेदित कर दिल क्षेत्र स रक्त एकत्र की गई। चूहों का रक्त स ट्रीपैनोसोमा की शुद्धि का लिए डी.इ.ए.इ. सेल्यूलोज़ क्रोमैटोग्राफी किया गया। फिनॉल क्लोरोफॉर्म निस्सारण और उसका बाद इथेनॉल अपघतन का द्वारा संग्रहित *ट्रीपैनोसोमा इवान्सी* की पेल्लेट्स (गोलियों) स डी.एन.ए. निष्कर्षण किया गया। पारंपरिक विधि का रूप में ट्रीजोल अभिकर्मक द्वारा और निर्माता का प्रोटोकॉल का उपयोग कर प्रोमेगा किट द्वारा कुल आर.एन.ए. का निष्कर्षण किया गया। क्लोनटेक आर.टी.-पी.सी.आर. किट का उपयोग कर कुल आर.एन.ए. स सी-डी.एन.ए. का संश्लेषण और उसका परिशोधन किया गया। *ओ.पी.डी.बी*, *पी.एफ.आर.1* और *पी.एफ.आर.2* जीन का वांछित एमप्लीकोंस को जीन विशिष्ट प्राइमरों का उपयोग करते हुए पी.सी.आर. द्वारा प्रवर्धित किया गया (*पी.एफ.आर.1* और *पी.एफ.आर.2* जीन का एमप्लीकोंस को आर.टी.-पीसीआर द्वारा भी प्रवर्धित किया गया)। प्रवर्धित पी.सी.आर. उत्पादों को इथीडीएम ब्रोमाइड स चिह्नित 1.2% अगारोस जेल पर विश्लेषण किया गया और *ओ.पी.डी.बी*, *पी.एफ.आर.1* और *पी.एफ.आर.2* जीन का आकार का आधार पर पहचान की गई। इलुस्टरा जी.एफ.एक्स. पी.सी.आर. डी.एन.ए. और जेल बैंड शोधन किट द्वारा 1% कम पिघलना वाला एगारोज जेल स अपेक्षित आकार का एमप्लीकोंस को शोधित किया गया। दिलचस्पी का डीएनए टुकड़ा को पी.जी.इ.एम.टी. आसान वेक्टर में क्लोन करना का लिए लायगेट किया गया और लायगेशन मिश्रण को *ई. कोलाई* जेएम.109 उपभेदों में तब्दील किया गया। रीकॉम्बीनैंट प्लाज्मिड युक्त कोशिकाओं को लुरीया बर्तानी अगार जिनमें एक्सगाल, आई.पी.टी.जी. और एम्पीसिलीन होता है, नीली/सफेद कॉलोनी का आधार पर पहचाना गया। *इको* आर1 का उपयोग कर प्लाज्मिड डी.एन.ए. का प्रतिबंध एंजाइम पाचन द्वारा रिकोम्बिनेंट्स की जाँच की गयी और पाया गया कि *ओ.पी.डी.बी* जीन का लिए 2092 बेस जोड़ी, *पी.एफ.आर.1* का लिए 1769 बेस जोड़ी और *पी.एफ.आर.2* का लिए 1767 बेस जोड़ी का

आसपास डी.एन.ए. के टुकड़े रिलीज हुए हैं। जीन विशिष्ट प्राइमरों की उपस्थिति में सीधे *ई. कोलाई* से प्लाज्मिड आवेषण के त्वरित जांच के लिए कॉलोनी पी.सी.आर. किया गया। *ओ.पी.डी.बी, पी.एफ.आर.1* और *पी.एफ.आर.2* जीन की क्लोन के पुष्टि के बाद प्लाज्मिड डी.एन.ए. का अनुक्रम किया गया और प्राप्त परिणामों के अनुसार *ओ.पी.डी.बी, पी.एफ.आर.1* और *पी.एफ.आर.2* जीन के कोडिंग अनुक्रम क्रमशः 2092 बेस जोड़ी, 1769 बेस जोड़ी और 1767 बेस जोड़ी के पाये गये। वंशावली और अनुक्रम विश्लेषण क्लसटल एक्स और मेगा 5 सॉफ्टवेयर के उपयोग के द्वारा किया गया। *ओ.पी.डी.बी, पी.एफ.आर.1* और *पी.एफ.आर.2* जीन की ट्री टोपोलॉजी (सांस्थिति) 100% बूटस्ट्रैप मूल्यों के साथ पड़ोसी शामिल होने और अधिकतम कृपणता की विधि पर आधारित है। *ओ.पी.डी.बी, पी.एफ.आर.1* और *पी.एफ.आर.2* जीन के प्राप्त प्रोटीन अनुक्रमों का एकाधिक अनुक्रम संरेखण इ.बी.याई. में क्लसटल डब्ल्यू (क्लसटल 2.1) के साथ किया गया। पहचान किये गये *ओ.पी.डी.बी, पी.एफ.आर.1* और *पी.एफ.आर.2* के जीन अनुक्रमों ने अन्य प्रकार के *ट्रायपैनोसोमा* और *लीशमैनिया* जीन अनुक्रमों के साथ एक करीबी अनुरूपता दिखाई। प्राप्त ओ.पी.डी.बी, पी.एफ.आर.1 और पी.एफ.आर.2 प्रोटीन के 3 डी संरचना मॉडल का निर्धारण अनुरूपता मॉडलिंग प्रोटोकॉल का उपयोग कर किया गया। ओ.पी.डी.बी प्रोटीन के अंतिम स्थिर संरचना में 9 सीट्स, 4 बीटा अल्फ़ाबीटा इकाई, 23 बीटा हेयरपिन्स, 11 बीटा बल्जेज, 39 असट्रेन्ड्स, 22 हेलिसेस, 20 हेलिक्स हेलिक्स इंटरफेसेस, 72 बीटा टर्न और 6 गामा टर्न पाये गये। पी.एफ.आर.1 के स्थिर संरचना में 4 सीट्स, 1 बीटा अल्फ़ाबीटा इकाई, 2 बीटा हेयरपिन्स, 2 बीटा बल्जेज, 9 असट्रेन्ड्स, 35 हेलिसेस, 57 हेलिक्स हेलिक्स इंटरफेस, 105 बीटा टर्न और 16 गामा टर्न और पी.एफ.आर.2 संरचना में 4 हेलिसेस, 4 हेलिक्स हेलिक्स इंटरफेस 3 बीटा टर्न और 2 गामा टर्न पाये गये।

1. INTRODUCTION

Trypanosomosis caused by protozoan parasite of the genus *Trypanosoma,* is a very serious and often fatal blood protozoan disease of domestic animals severely limiting their productivity in the subtropical and tropical regions of the world. *Trypanosoma* belonging to the Family *Trypanosomatidae* is parasitic in the blood, lymph and tissues of invertebrates and vertebrates, including humans. Most species live a part of their life cycle in the intestine of insects and other invertebrates, the flagellate stage being found only in the vertebrate host. The difference in species depends on size and shape of the body, position of nucleus, degree of development of the undulating membrane and flagellum (Smyth, 1996). Different species of *Trypanosoma* infect cattle, sheep, goats, pigs, horses, donkeys, camels, many wild life species and man. Acute infection causes a severe febrile illness characterized by coughing, dyspnea, pulmonary edema, idiopathic cardiomyopathy, hepato-splenomegaly and immunosuppression, and death by respiratory distress or cardiac failure (Hoare, 1972; Brun *et al*., 1998). The most significant impact probably comes from chronic trypanosomosis resulting in infertility, reduced milk yield, reduced weight gain and lower work output, contributing to the lower provision of animal proteins and lower yield of agricultural products due to lack of working livestock (Boid *et al*., 1996).

Trypanosomosis caused by *Trypanosoma evansi*, is the most pathogenic parasitic disease of camelids in all camel rising countries causing high morbidity and mortality (Luckins, 1992). This parasite has a wide range of distribution throughout tropical and sub-tropical regions of the world. *T. evansi* was reported originally from India, where the term 'surra' is used to describe the disease. Chronic infection of *T. evansi* may be present for three or more years in camels and this form of the disease is commonly known as “Tebersa”. In South Africa, another form of trypanosomosis is prevalent which is known as "nagana". *T. evansi* perhaps evolved from *T. brucei,* when camels entered the tsetse fly belt and acquired infection. Later, the disease was maintained mainly through mechanical transmission by biting flies notably, the *Glossina species* (Tseste-fly), *Tabanus species*, *Haematobia,*

Stomoxys, *Chrysops, Lyperosia* and *Pangonia* and spread to the Northern Africa, Middle East, India and the Far East Asian countries (Lukins, 1992).

T. evansi consists of large number of morphologically identical populations that differ significantly in various biological characteristics such as host range and pathogenicity. In India, the disease impact varies in different hosts from region to region. Among the host range, horses and camels are mainly affected, followed by cattle, buffaloes, dogs, goats, sheep and pigs (Gill, 1991). The disease in camels is very common and outbreaks frequently occur during and after rainy season though sporadic cases are met with throughout the year (Pathak & Khanna, 1995).

Control of trypanosomosis is principally based on insecticide spraying to control the vector population and on regular treatment of livestock at risk in endemic area through chemoprophylaxis and chemotherapy. The high cost of regular drug and insecticidal treatment, the limited effectiveness of insecticides application in high rainfall areas, the possibility of environmental pollution by insecticides, the increasing incidence of parasite resistance to available drugs and absence of new drugs to replace them are the major problems that make vector and trypanosomosis control difficult and expensive. The disease has been contained mainly through chemotherapy but increasing drug resistance challenges the application of drug-based control strategies. There is no effective vaccine available due to variations of surface proteins as antigens between and within *Trypanosoma* species. Variant surface glycoprotein coat of *Trypanosoma evansi* is changeable when host forms antibody against it. *T. evansi* possesses about 1000 VSG genes; only one gene is active at a time (Janz and Clayton, 1994). Vaccine development against animal trypanosomosis based on variant surface glycoprotein is totally abandoned (Donelson *et al*., 1998). This has prompted researchers to look into alternative invariant proteins like protozoan proteases and paraflagellar rod proteins.

Proteases are a ubiquitous group of enzymes that play key roles in the life cycle of parasites, in the host-parasite relationship, and in the pathogenesis of parasitic diseases. Furthermore, proteases are targets for the development of new anti-parasitic therapy. Protozoan proteases play crucial role in the host – parasite interaction, and their characterization contributes to the understanding of the protozoan disease mechanisms. Proteases are important for parasitic survival; they are involved in the digestion of exogenous proteins for nutritive purposes (Rosenthal, 1999), invasion of host cells and tissues (Roggwiller *et al.,* 1996), and modification of host proteins (Caler *et al*., 1998).

The development of effective drugs and vaccines for protozoan parasitic diseases is hampered by a lack of understanding of the pathogenesis of these diseases and this is exacerbated by the complexity of parasite - host interactions. The identification of peptidases/proteases in protozoan parasites that are able to process regulatory host peptides under physiological conditions gave clues to possible mechanisms of parasite virulence that can be targeted to prevent these debilitating diseases. Oligopeptidase B (OpdB), one of such proteases, which is increasingly being implicated as an important virulence factor in trypanosomosis (Burleigh and Woolsey, 2002) and becomes the therapeutic target. Elucidation of the substrate specificity and regulation of OpdB activity paved the way to develop drugs that are specific for the parasite. Further, structural and functional characterization of OpdB in kinetoplastid protozoan parasites should aid in clarifying its physiological function and lead to the development of chemotherapeutic drugs for diseases that affect millions of people and livestock world-wide (Coetzer *et al*., 2008).

In *T. evansi* infections Oligopeptidase B has a major role to play in manifestation of disease. It is shown to proteolytically cleave many of the host derived peptides and proteins like kinogen, atrial natriuretic factor in bloodstream of infected host etc. (Morty *et al*., 2005a). Inability to inhibit this cysteine peptidase by host derived protease inhibitors makes Oligopeptidase B important during pathogenesis, thus making it an attractive drug target and basis for vaccine development.

The first OpdB was isolated from *Escherichia coli* and was called protease II. The presence of OpdB was shown in eukaryotes with the isolation of a peptidase with protease II-like activity from the South American trypanosome, *Trypanosoma cruzi* that causes Chagas disease in humans. This peptidase was first called alkaline peptidase and later Tc 120 proteinase, 120 kDa alkaline peptidase or *T. cruzi* OpdB and is distinct from the post-proline cleaving 80 kDa prolyl oligopeptidase of *T. cruzi*, POP Tc80. Oligopeptidase B was also isolated from the African trypanosomes, *T. brucei brucei* and *T. congolense*, that cause nagana in cattle. The peptidases were named oligopeptidase from *T. brucei* (OP-Tb) and oligopeptidase from *T. congolense* (OP-Tc). The genes encoding the *T. cruzi*, *T. b. brucei* and *T. evansi* oligopeptidases were sequenced and named the opdB gene, and its products were named oligopeptidase B ‘‘basic amino acid specific oligoendopeptidase’’ (Yoshimoto *et al*., 1995).

The *Trypanosoma evansi* genes Paraflagellar Rod 1 (*pfr1*) and Paraflagellar Rod 2 (*pfr2*), which encode the major structural components of the paraflagellar rod (PFR) are also highly pathogenic genes and restricted to the flagella of kinetoplastids. In trypomastigote forms, and to some extent in epimastigote forms of trypanosomes, the flagellum is attached along the cell body. In such cases, the proximal domain of the PFR is linked via filaments to the inner face of the flagellar membrane and then to the Flagellum Attachment Zone (FAZ). The PFR and axoneme maintain a precise orientation in regard to each other with the central pair microtubules having a consistent position (Gadelha *et al*., 2006).

The genome sequencing projects for *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* now showed that the genes encoding *pfr1* and *pfr2* were distinct but related and were present in separate tandem arrays. Sequence analysis showed that both proteins were the result of a single gene duplication event that predated the divergence of Euglenida and Kinetoplastida and that all known extant instances of these proteins fit into a simplified and consolidated PFR1 and PFR2 nomenclature. The

major PFR proteins already described in *T. brucei*, *T. cruzi*, *Leishmania mexicana* and *L. major* should be numbered on the basis of molecular mass, with the protein of higher molecular mass being numbered PFR1 (Gadelha *et al*., 2004).

One of the most unique structural features of the trypanosome flagellum is the presence of a large para-crystalline filament, the paraflagellar rod, which extends alongside the axoneme from the flagellar pocket to the flagellum tip. PFR is vital for trypanosome motility (Bastin *et al*., 1998) and is unique among the kinetoplastids as their heteropolymers provide the building blocks of flagellum (Abdille *et al*., 2008a). The Kinetoplastida PFR is a complex, trilaminar lattice-like structure with proximal, intermediate and distal domains defined. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. Both highly abundant proteins PFR1 and PFR2 are considered as the classic defining components of the PFR. More than 40 additional proteins have been found associated with the PFR through biochemical, bioinformatic and immunological techniques. The nature of these components provides increasing evidence for a PFR role in metabolic, regulatory and signalling functions.

PFR structures have been described for three large groups of flagellates: the kinetoplastids, the euglenoids, and the dinoflagellates (Cachon *et al*., 1988). They are always highly ordered lattices of fibrous proteins that are located inside the flagellum and assume a fixed orientation with respect to the microtubular axoneme (Souto-Padron *et al*., 1984). This unique structure is found only in these organisms, and its protein components are structurally and immunologically distinct from any of the major filamentous systems of the host cell, including microfilaments, microtubules, or intermediate filaments.

The identification and research into new drug targets for trypanosomosis is necessary, and the development of novel, more effective, and less toxic drugs is an urgent priority. Due to lack of understanding of the pathogenesis of trypanosomosis,

development of effective drugs and vaccines for this protozoal disease is hampered and this becomes worse by the complexity of parasite - host interactions. Oligopeptidase B is emerging as an important virulence factor and therapeutic target in *Trypanosoma evansi* infection. The PFR is a functionally important structure that is present in pathogenic trypanosomatids but absent from their mammalian hosts. As such, the PFR is a logical target for therapeutic intervention in the infection process. Promising inroads in this area have been made using PFR proteins as the basis for a vaccine.

DNA sequencing may be used to determine the sequence of individual genes, larger genetic regions (i.e. clusters of genes), full chromosomes or entire genomes. Sequencing of *opdB*, *pfr1* and *pfr2* genes of *T. evansi* may be useful to identify the function of these genes and encoding proteins. Cloning of the purified PCR product of a gene is essentially required for the replication of DNA molecules to generate a large population of cells containing identical DNA molecules. Once the large amount of DNA molecules is isolated they can be used for sequencing of nucleic acid of the gene. In this manner a complete or partial sequence of the protein which is encoded by available gene can also be confirmed. Sequence analysis of the gene and its protein is the most appropriate method for the confirmation of specificity of the target region of any gene. Sequence of the gene can be compared with those of known genes to try to derive a function for that gene.

Keeping in view of above facts, the detail molecular study of Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *Trypanosoma evansi* was undertaken in present study with following objectives:

1. **To isolate Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *Trypanosoma evansi* from camel by polymerase chain reaction and clone the amplicons**

2. **Characterization of above genes through sequencing**

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3. MATERIALS AND METHODS

3.1. Material

3.1.1. Experimental organism

For isolation of Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes, the experimental organism is *Trypanosoma evansi*, a blood protozoa included in Phylum *Sarcomastigophora*, Sub-phylum *Mastigophora*, Class *Zoomastigophorea*, Order *Kinetoplastida* and Family *Trypanosomatidae*. It is an important parasitic protozoon of camelids causing high morbidity and mortality.

3.1.2. Equipments

- Refrigerated micro centrifuge - Hettich, model- Mikro 22 R, Germany
- Refrigerated high-speed centrifuge - Biofuge Primo R, Heraeus, Germany
- Single Pan digital balance - Precisa, 125 A SCS, Switzerland
- Water Bath cum shaker - Aqua shake, Kuhner, Switzerland
- Incubator Shaker - Lab-Therm, Kuhner, Switzerland
- Trans illuminator - UVP, USA
- Micropipettes: 2.5, 10, 20, 100, 200 and 1000 µl capacity – Eppendorf, Germany and Nichipet, USA
- Horizontal Agarose Gel Electrophoresis apparatus with power supply - Genei, India
- Ultra low freezer (-80°C) - Model U410, NBS, USA
- Deep Freeze (-20°C) - Heto, Denmark
- Microwave oven - Kenstar Electronic Ltd., India
- Dry heating block - Thermocon, Genei, India
- Cyclo mixer– CM 101, Remi, India
- Magnetic Stirrer– Remi, India
- pH meter– µ pH system 361, Systronics, India
- Incubator– Model BD-53, Binder, USA
- Horizontal laminar air flow – Telstar, AV- 30/70, USA
- Thermocycler – Mastercycler Gradient, Eppendorf, 5330, Germany

- Gel Documentation System – AlphaImager 2200, USA
- Water Purification System – Elix-Milli-Q, Synthesis Type, Millipore, USA
- UV- vis Spectrophotometer– UV mini 1240, Shimadzu, USA
- Trinocular Compound microscope, Nikon, USA

3.1.3. Chemicals

- Trizol (Invitrogen, USA)
- Ampicillin, Bovine serum fraction-V (BSA), Bromophenol blue, RNAse-A, Sodium dodecyl sulphate (SDS), Sucrose and Xylene cyanol F.F. were from Sigma Chemicals Co., St.Loius, USA
- DNAse (New England Biolabs, USA)
- DNA purification kit and Plasmid isolation kit (GE healthcare, USA)
- Proteinase K, Tris Base, Ethidium bromide, (Sigma Chemicals Co., St.Loius, USA)
- dNTPs (Larova GmbH, Germany and from Promega, USA)
- Tryptose Phosphate Broth (TPB) were from HiMedia (India)
- Glycerol and other biochemicals were from Sigma Chemicals Co., St.Loius, USA
- DNA molecular size markers were from Invitrogen, USA
- RNA isolation Kit from Promega, USA
- cDNA synthesis kit from Clontech, USA
- PVDF membranes were from Millipore, India

- Agarose – Analytical and Preparative grades, Peptone and Yeast Extract were from Invitrogen, USA.
- Seakem Low Melting Point Agarose from Lonza, Switzerland
- Other chemicals of analytical grade were either from Sisco Research Laboratories India, Glaxo Laboratories India Ltd., or E.Merck (India) Ltd.

3.1.4. Glass and plastic ware

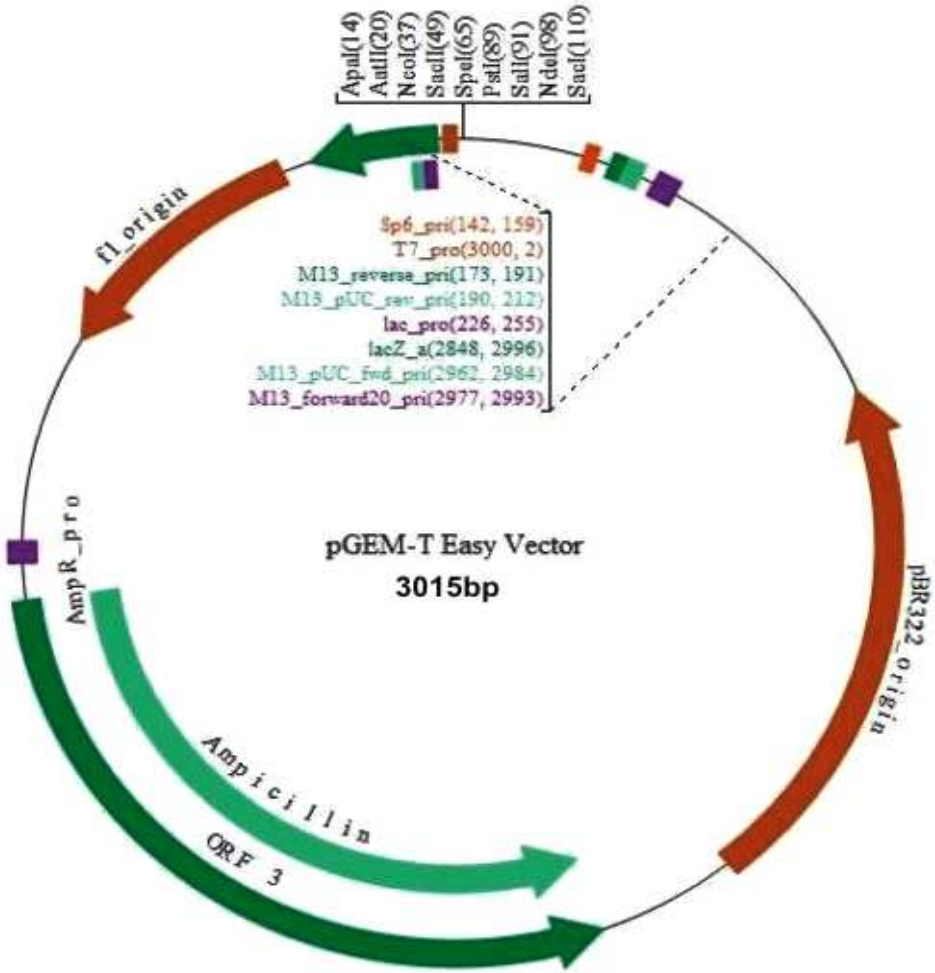
Glassware used in this study was procured either from Borosil India Ltd., or from Duran Schott, Germany. All the Glassware were thoroughly washed and sterilized as per standard protocols before use. Micro centrifuge tubes and micropipette tips were either from Axygen or Eppendorf, India Ltd.

3.1.5. Media and buffers

Composition of media, buffers and solutions used in this study is given in the Appendix No. I - III or at appropriate places.

3.1.6. Vector

pGEM- T Easy vector (Promega) available in the laboratory was employed. The vector was propagated in *Escherichia coli*, JM109 cells, extracted and used for cloning studies. The map of the pGEM- T Easy vector is shown in plate 1.


ApaI(14)
AatII(20)
NcoI(37)
SacII(49)
SpeI(65)
PstI(89)
SalI(91)
NdeI(98)
SacI(110)
f1_origin
Sp6_pri(142, 159)
T7_pro(3000, 2)
M13_reverse_pri(173, 191)
M13_pUC_rev_pri(190, 212)
lac_pro(226, 255)
lacZ_a(2848, 2996)
M13_pUC_fwd_pri(2962, 2984)
M13_forward20_pri(2977, 2993)
pGEM-T Easy Vector
3015bp
AmpR_pro
Ampicillin
ORF 3
pBR322_origin

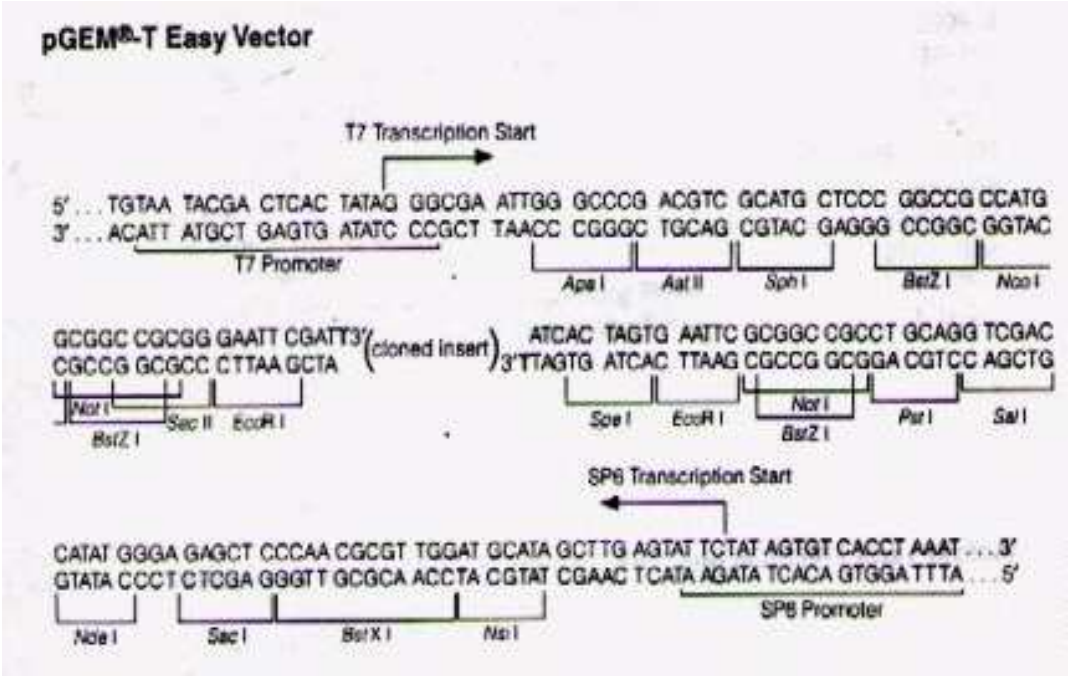


Plate 1. Map of the pGEM- T Easy vector

3.1.7. Host Systems

Escherichia coli strains JM109 (Promega) stored at –70^{0}C was used for propagation of plasmids.

3.1.8. Primers

Gene specific primers for *pfr1* and *pfr2* genes were designed from published sequences (Accession No. EU366960 for *pfr1* and Accession No. EU258755 for *pfr2* gene) using the primer designing tool at NCBI. For *opdB* gene already designed primer sequences by Morty *et al*. (2005a) were used. All the primer sequences were stretched 5' to 3' end. Lyophilized form of the primers were obtained from Clontech, USA, Eurofins, India and GCC Biotech., India reconstituted as 100 mmol. stock in sterile TE buffer. Primers at working concentrations of 10 pmol in sterile TE buffer were used for PCR amplification.

Table 3.1: Primer sequences used to amplify *opdB*, *pfr1* and *pfr2* genes

Genes of *T. evansi*	Forward and reverse Primers (5' to 3')
opdB	**F** 5'GGACACATATGATGCAAACTGAACGTGGTCC3' **R** 5'TACGCTCATATGCTACTTCCGCAGCAGCGGCC3'
pfr1	**F** 5'ATGGCCGCAGTTGACGATGCCAC3' **R** 5'CTATTCGAGGCGTGCCGGTG3'
pfr2	**F** 5'GCAGAATTCATGAGCGGAAAGGAAGTTGAA3' **R** 5'GACGGTACCCTGAGTGATCTGCGGCATC3'

3.1.9. Enzymes

Restriction enzyme *Eco*R1 and *Taq* DNA polymerase were used from Promega, USA.

3.2. Methods

During the present study attempts were made to identify the Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *T. evansi* from camel (*Camelus dromedarius*). The procedure followed has been described in details as under:

3.2.1. Identification of *Trypanosoma evansi* infected camel

Initially, some trypanosomosis suspected camels were identified in the National Research Centre on Camel, Bikaner (Rajasthan). Blood smear from suspected animals was prepared and stained with Geimsa stain after proper fixation with methanol (Appendix-II.1). Properly stained blood films were examined under compound microscope to confirm the infection of *T. evansi* in the camels. After confirmation of *T. evansi* isolates, blood from infected host was collected. For this 5 ml blood was collected from the jugular vein using 9 ml vaccutainer tube containing EDTA (ethyl diamine tetra acetic acid). 0.5 ml blood (with the help of insulin syringe) was inoculated intraperitoneally into the each experimental animal which were Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner).

3.2.2. Propagation of trypanosomes

Based on the infectivity titration as given by Lumsden *et al*. (1973), a convenient passage dose/interval for mice for each of the isolates of *T. evansi* was arrived at and routinely used throughout the investigation. The method consisted of examination of wet blood films from peripheral blood of the laboratory hosts (mice/rats) and scoring the degree of parasitaemia. The following scheme described by Desowitz and Watson (1951) was followed for interpretation of degree of parasitaemia:

S : Scanty infection i.e. less than one trypanosome per microscopic fields

+ : Average 1-5 trypanosomes per microscopic fields

++ : Average 6-10 trypanosomes per microscopic fields

+++ : Average 11-20 trypanosomes per microscopic fields

++++ : Average more than 20 trypanosomes per microscopic fields

M : Massive infection i.e. trypanosomes equal or exceeding the number of erythrocytes in the field

The estimation of the working/infectivity dose for routine passage in mice was then arrived by dilution of the suspension of trypanosomes collected through tail blood (++++ or M).

3.2.3. Collection of isolates

The blood of mice was collected from heart region in 5 ml disposable syringe containing 0.1 ml heparin solution after dissecting the mice which had massive infection.

3.2.4. Purification of trypanosomes

DEAE (Diethyl amino ethyl) cellulose column chromatography method was used for purification of trypanosomes (Lanham and Godfrey, 1970). This technique resulted in a suspension of trypanosomes that was free of any other figurative elements. This technique was based on the electrostatic characterization of DEAE- cellulose at a given pH, to fix figurative blood elements (white and red cells, platelets) and not trypanosomes.

3.2.4.1. Preparation of column

Purification of trypanosomes was essentially needed to isolate the total cellular RNA/genomic DNA and further amplification, cloning and sequencing purposes.

10 grams diethyl amino ethyl (DEAE) cellulose powder was suspended in 0.1M NaOH for 1h with intermittent stirring. The cellulose was then washed repeatedly with distilled water until the pH of the supernatant was neutral. The sediment was then mixed with an equal volume of 0.1M HCl for 1h and thereafter, washed with distilled water as described previously until the pH of the supernatant was 6.8-7.0. The slurry was stored at 4^0C until used. The slurry was equilibrated with PSG (Phosphate saline glucose) buffer (pH 8.0, Appendix–I.3) supplemented with glucose at 1% level by repeated washings. The slurry was then packed carefully to a column height of 10 cm (2.5 cm diameter) fitted with a sintered glass disc of zero porosity. The flow of elute was controlled by Teflon screw clamp fitted to the column outlet. The column gel was further equilibrated by passing PSG buffer (pH 8.0) until the pH of the eluent was 8.0.

3.2.4.2. Charging of column

Collected blood was diluted with 1:3 chilled PSG (Phosphate saline glucose) buffer (pH 8.0, Appendix–I.3) before application to the column. The diluted blood was then carefully charged through the sides of the column of the gel surface and after the blood sample entered the bed, small quantities of PSG buffer (pH 8.0) were applied. Drops of eluted product from column were examined time to time under the microscope to spot the separated trypanosomes. The trypanosomes were collected in a beaker and were pelleted by centrifugation at 1000 rpm for 10 min. The separated trypanosomes were pooled and were either resuspended in PBS (Appendix–I.2) for use in various applications or the pellet was kept at -20^{0}C for further processing.

3.2.5. Isolation of genomic DNA from pellets of *Trypanosoma evansi*

DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the method utilized by Desquesnes and Davila (2002) for the preparation of animal trypanosomes DNA from plane blood. The procedure for DNA isolation was same as the most commonly used procedure for DNA isolation from blood suggested by Sambrook and Russel (2001) and involving four major steps:

- Lysis of *Trypanosoma evansi* pellet by using the Proteinase K buffer (Appendix–I.4), Proteinase K Enzyme and RNase A.
- Digestion of proteins.
- Extraction of DNA with Phenol and Chloroform.
- Precipitation of DNA with Alcohol.

Collected pellet of *Trypanosoma evansi* was taken in an eppendorf tube and 500 µl PK Buffer, 50 µl PK Enzyme and 20 µl RNase A were added into it and mixed gently. After proper mixing it was incubated at 56ºC for 3 to 4 hours, during incubation tapping was done time to time. After incubation 500 µl of phenol: chloroform: Isoamyl alcohol (25: 24: 1) was added to above

eppendorf tube and mixed well. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper phase was transferred to a fresh eppendorf tube and treated with equal volume of phenol: chloroform: Isoamyl alcohol (25: 24: 1) and mixed well. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature. Then transferred the upper phase into a clean eppendorf tube and 500 µl chilled chloroform was added. After proper mixing the mixture was again centrifuged at 10,000 rpm for 10 minutes at room temperature. Upper phase was transferred in to a clean eppendorf tube and 10µl sodium acetate (3M) and 250 µl chilled ethanol (0.1 volume 3M sodium acetate and 2.5 volume ethanol) were added in it and then kept at -20°C for overnight. Next day, above eppendorf tube was centrifuged at 13, 000 rpm for 10 minutes at room temperature. The upper phase was discarded and the DNA pellet washed by 500 µl of 70% chilled ethanol then centrifuged it at 13, 000 rpm for 10 minutes at room temperature. Supernatant was poured off and the pellet was dried by Dry Bath and dissolved this DNA pellet in 50 µl of TE Buffer. Then the concentration and purity of DNA sample was determined.

3.2.6. Quantitative and Qualitative assessment of DNA

3.2.6.1. Spectrophotometric Determination

For quantifying the amount of DNA, O.D. values were recorded at wavelengths of 260 and 280 nm (The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample and an O.D. of 1 corresponds to approximately 50 µg/ml for the double stranded oligonucleotides).

The DNA was diluted with TE Buffer at the ratio of 1: 50 and mixed accordingly. TE buffer was taken as a blank and respective O.D. was recorded to calibrate at zero.

After setting spectrophotometer with blank the O.D. values at 260 nm and 280 nm were recorded for DNA and protein, respectively. The concentration of unknown double stranded DNA sample was estimated, using the following formula:

DNA concentration (µg/ml) = Absorbance at 260nm x dilution factor x50 = A1 X 50 X 50

The ratio between the readings at 260 nm and 280 nm (O.D. 260/280) provides estimates of purity of nucleic acid. The ratio of O.D. values of high quality DNA ranged between 1.8 and 2.

A1 / A2 = Absorbance at 260/280 = DNA/protein = 1.85

3.2.6.2. Checking of quality of DNA

The genomic DNA isolated from the *T. evansi* was checked for quality, purity and concentration. Only the DNA samples of good quality were used for further analysis.

Horizontal submarine agarose gel electrophoresis was carried out to check the quality of genomic DNA using 0.8 % w/v agarose. At the start the gel casting tray was prepared by sealing it's both end with adhesive tape and then the comb was set over it in away to keep a gap of at least 0.5 mm between the tips of comb teeth and floor of the casting tray, so that the wells got completely sealed by agarose.

Subsequently, 0.8% agarose (w/v) suspension in 1 X TAE buffer was made and heated on an electric heater or in microwave until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling it to about

50^0C, ethidium bromide (10 mg/ml) @ 5 µl per 100 ml of agarose solution was added to a final concentration of 0.5 µg/ml and was mixed gently. The agarose solution was poured into the sealed casting tray. The gel was prepared to about 4 mm thicknesses. The agarose gel was allowed to set completely at 4^0C temperature before the comb was gently removed. The adhesive tape was also detached and gel casting platform was submerged in the electrophoresis tank containing 1 X TAE buffer.

For loading the samples, 10 µl of autoclaved triple distilled water was mixed with 5 µl DNA. 1 µl of 6 X gel loading dye (Appendix-II.2) was mixed with 5 µl diluted DNA for loading in each well of gel. Electrophoresis was performed at 4V/cm (60-70V) for ~1 hour after loading the DNA sample into the well. Once the electrophoresis was over, the gel was visualized under UV trans-illuminator and documented by photography. Only DNA sample showing intact bands were used for further analysis.

3.2.7. Extraction of total RNA

Extraction of total RNA was done by Trizol reagent as conventional method suggested by Sambrook and Russel (2001) as well as using Promega SV Total RNA isolation kit following the manufacturer protocol.

3.2.7.1. Extraction of total RNA by Trizol reagent

Purified *T. evansi* pellet was dissolved in 1 ml PBS and 0.5 ml amount of Trizol reagent was added into it and mixed gently then incubated at room temperature for 5 min. The mixture was centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a fresh microcentrifuge tube and treated with equal volume of chloroform by vortexing. The mixture was centrifuged at 12,000 rpm for 10 min. The aqueous phase was collected and precipitated with equal volume of isopropanol in the presence of

0.3M Ammonium acetate and subsequent incubation at room temperature for 1 hour. The above mixture was centrifuged at 12,000 rpm for 10 min and the RNA pellet thus obtained was washed once with 1 ml of 70% alcohol (Appendix–III.2), dried under vacuum and resuspended in 10 µl of di ethyl pyro carbonate (DEPC) treated and filtered Milli Q water and stored at –70^{0}C until further use.

3.2.7.2. RNA isolation by Promega SV Total RNA isolation Kit

RNA isolation was done by using Promega SV Total RNA isolation system involved following steps:

3.2.7.2.1. Cell lysis

One *T. evansi* cell pellet (around 30 mg) was taken to which 175 µl of RNA lysis buffer (RLA) were added. This was mixed thoroughly by inversion. 350 µl of RNA dilution buffer (RDA) was taken in fresh autoclaved RNAase-free eppendorf tube then lysate was added. Mixed by gentle inversion for 3-4 times. Placed in heating block at 70°C for 3 minutes. Centrifuged the mixture for 10 minutes at 14000 x g and cleared lysate was transferred to a fresh eppendorf tube. 200 µl of 95% ethanol was added to the cleared lysate, and mixed by pipetting 3-4 times.

3.2.7.2.2. Filtration and RNA binding

Lysate and ethanol mixture was transferred to the spin column assembly and centrifuged at 14000 x g for one minute. Spin basket was taken from spin column assembly, and the eluted liquid was discarded. 600 µl of RNA wash solution (diluted with ethanol) was added to the spin column assembly and centrifuged at 14000 x g for 1 minute. DNase incubation mix was prepared in a sterile tube by combining 40 µl yellow core buffer, 5 µl 0.09 M $MnCl_2$ and 5 µl of DNase I enzyme. After proper mixing by pipetting, 50 µl of DNase incubation mix was applied directly to the membrane inside the spin basket and incubated 15 minutes at

room temperature. After incubation 200 µl DNase stop solution (DSA ethanol added) was applied to the spin basket and centrifuged at 14000 x g for 1 minute.

3.2.7.2.3. Washing

600 µl of RNA wash solution (RWA ethanol added) was added to the spin basket and centrifuged at 14000 x g for 1 minute. Eluted liquid was discarded and 250 µl of RNA wash solution was again added to the spin basket. Centrifuged at 14000 x g for 2 minutes and elution tube with eluted liquid was discarded.

3.2.7.2.4. Elution of RNA

Spin basket was transferred to fresh elution tube and 100 µl of Nuclease-free water was added to membrane then centrifugation was done at 14000 x g for 1 minute to elute the RNA. Eluted RNA was transferred to fresh sterile eppendorf tube and stored at – 80°C for future use.

3.2.7.3. Qualitative confirmation of RNA

The presence of RNA was confirmed by using gel electrophoresis (Sambrook and Russel, 2001). Appropriate quantity of agarose (Analytical grade) was boiled in 30 ml of 1x TAE buffer (Appendix–I.1.1)) to obtain uniform molten agarose (of desired 1.2 %, Appendix–II.5) which was cast in appropriate gel-casting tray fitted with acrylic comb and left for setting. Prior to casting the gel, the molten agarose was allowed to cool to about 50^{0}C after which ethidium bromide (Appendix II.3) was added to make a final concentration of 0.5 µg/ml and mixed thoroughly. The acrylic comb was carefully removed after the gel had set perfectly. The tray with gel was then transferred to and submerged in an electrophoresis tank containing 1x Tris Acetate EDTA (TAE) buffer.

RNA to be analyzed was mixed with appropriate volume of 6x DNA loading dye (Appendix–II.2). Electrophoresis was carried out at 5V/cm until the tracking dye (6x DNA loading dye) had just passed out of the gel. The RNA bands were visualized under UV illumination and documented.

3.2.8. cDNA synthesis and purification

cDNA synthesis was done using Clontec RT-PCR Kit.

3.2.8.1. Protocol for cDNA synthesis

In a sterile 0.5 ml microcentrifuge tube, 12.5 µl RNA (with nuclease free water) was taken. 1.0 µl of oligo $(dT)_{18}$ primer was added to RNA. RNA mix was placed in heating block for 2 minute at 70°C then placed it rapidly on ice before proceeding to the next step. Various components such as 5X reaction buffer 4.0 µl, dNTP mix (10mM each) 1.0 µl, Recombinant RNase inhibitor 0.5 µl and MMLV reverse transcriptase 1.0 µl were added to RNA mix. Components of the tube were mixed by pipetting up and down. Reaction mix was incubated at 42°C for 1 hour in thermo cycler. Then reaction mix was heated at 94°C for 5 minutes in thermo cycler to stop the cDNA synthesis reaction and to destroy any DNase activity. After spin down the components of the tube the cDNA was stored in -80°C.

c-DNA quality was also analyzed by using agarose gel electrophoresis as describe earlier.

3.2.9. Amplification of *opdB*, *pfr1* and *pfr2* genes by PCR/RT-PCR

Principle of PCR

The purpose of PCR (polymerase chain reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

3.2.9.1. The cycling reaction:

There are three major steps in a PCR, which are repeated for 30 to 40 cycles. This is done on an automated thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

3.2.9.1.1. Denaturation at 94°C

During the denaturation the double strand melts and opens to single stranded DNA after all enzymatic reaction.

3.2.9.1.2. Annealing

The primers are anneal around, annealing being caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primers and the single stranded template.

The more stable bonds last a little bit longer (primers) that fit exactly on the little piece of double stranded DNA (primers and template.), the polymerase can attach and start copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer that does not break any more.

3.2.9.1.3. Extension at 72 °C

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have to a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with on exact match get loose again (because of higher temperature) and don't give an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase add dNTP's from 5' to 3', reading the template from 3' to 5'side, bases are added complementary to the template). Because both of strands are copied during PCR there is exponential increase of the number of copies of the gene.

3.2.9.2. Optimization of PCR Parameters

Various combinations of reaction chemical were tried to optimize the concentration of each component. The PCR parameter, viz. annealing temperature [2(A+T) +4(G+C)] and cycling conditions were optimized to obtain a specific amplified product in sufficient quantity. The reaction volume was kept constant at 50 µl. 4 times volume of reaction mixture was prepared as master mix, after proper mixing 50 µl reaction mixture was divided in four PCR tubes. For RT-PCR of *pfr1* and *pfr2* genes one more reaction mixture was made as control without target c-DNA. The standardized concentrations of components used in the reaction mixture are given in Table 3.2 – 3.4.

Table 3.2: PCR reaction mixture for *opdB* gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	4 µl	2.5 mM of Mg^{2+}
Primer F	0.25 µl	10 pM
Primer R	0.25 µl	10 pM
Template DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	33.75 µl	-
Total volume	**50 µl**	-

Table 3.3: PCR reaction mixture for *pfr1* gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP

$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	0.25 µl	10 pM
Primer R	0.25 µl	10 pM
Template DNA/c-DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	34.75 µl	-
Total volume	**50 µl**	-

Table 3.4: PCR reaction mixture for *pfr2* gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	0.20 µl	10 pM

Primer R	0.20 µl	10 pM
Template DNA/c-DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	34.85 µl	-
Total volume	**50 µl**	-

PCR amplification was carried out in thermal cycler using the optimized condition as given in table 3.2 - 3.4.

3.2.9.3. Protocol for amplification of *opdB*, *pfr1* and *pfr2* gene of *T. evansi* by PCR and *pfr1* and *pfr2* gene of *T. evansi* by RT-PCR

At first, a PCR master mixture, containing all the reaction components except genomic DNA/c-DNA, was prepared in ice under sterile condition. Care was taken to add *Taq* DNA polymerase at the end of preparation. After addition of all the components, the master mix was mixed gently, followed by spinning by table top micro centrifuge. Then, 49.5µl of master mix was added to each pre labeled PCR tubes (eppendorf tube) of 0.2 ml capacity. Finally, 0.5µl of good quality genomic DNA/c-DNA was added to each tube. For RT-PCR of *pfr1* and *pfr2* genes master mix was kept in one more tube without c-DNA as control. The contents of tube were mixed gently and spinned at 9000 rpm at 10 sec. Finally, the PCR tubes were arranged in a preprogrammed thermo cycler. PCR products obtained, after the completion of the programme, were kept at 4^0C in refrigerator for further analysis.

3.2.9.4. PCR programme

Several combinations of PCR programmes were tried before finalizing one programme giving the best amplification of the desired fragments. The standardized programmes for different genes are given below.

Table 3.5: PCR conditions for *opdB* gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	94°C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94°C 57°C 72°C	 30 sec. 1 min. 1 min. and 30 sec.	Over all total 35 cycle
III. Final extension	72°C	9 min.	One
IV. Hold	4°C		
Thermal Cycler lid temperature = 105°C			

Table 3.6: PCR conditions for *pfr1* gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	$94^{o}C$	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 $94^{o}C$ $63^{o}C$ $72^{o}C$	 30 sec. 1 min. 1 min. and 30 sec.	Over all total 35 cycle
III. Final extension	$72^{o}C$	9 min.	One
IV. Hold	$4^{o}C$		
Thermal Cycler lid temperature = $105^{o}C$			

Table 3.7: PCR conditions for *pfr2* gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	94°C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94°C 57°C 72°C	 30 sec. 1 min. 1 min.	Over all total 35 cycle
III. Final extension	72°C	10 min.	One
IV. Hold	4°C		
Thermal Cycler lid temperature = 105°C			

3.2.9.5. Analytical Agarose Gel Electrophoresis

PCR amplified DNA was analyzed by analytical agarose gel electrophoresis as per the procedure described by Sambrook and Russel (2001). The procedure of electrophoresis was same as mentioned in qualitative confirmation of RNA. DNA to be analyzed was charged into wells of gel alongside DNA molecular weight marker for the confirmation of molecular size of the DNA bands in relation to molecular weight marker.

3.2.9.6. Elution of DNA from low melting point agarose gel (preparatory)

DNA required for various manipulations was purified after electrophoresing the DNA in preparatory agarose gel. The method of gel preparation and casting were essentially the same as was done with the analytical method except for the usage of 1% Low Melting Point (LMP) Agarose in place of the analytical grade used earlier. The DNA was electrophoresed at a constant low voltage of 4mV/cm to facilitate clear separation of the DNA bands. The agarose slice containing the DNA band of interest was carefully excised from the gel under UV illumination with the help of a sterile scalpel and used for elution of the DNA within.

3.2.9.7. DNA purification (illustra GFX PCR DNA and Gel Band Purification Kit) method

DNA from LMP agarose slices was purified using illustra GFX PCR DNA and Gel Band Purification Kit involving the following steps

3.2.9.7.1. Sample capture

Capture buffer type 3 was added to the weighed agarose gel slice accordance to the weight of agarose slice. It was mixed by inversion and incubated at 60^0C for 15-30 minutes until the agarose was completely dissolved. When the agarose was completely dissolved then colour of capture buffer type 3-sample mix was checked and that was yellow. Then it was centrifuged for collecting the liquid at the bottom of the tube.

3.2.9.7.2. Sample binding

One GFX Micro spin column was placed into one collection tube. Then up to 800 µl of capture buffer type 3-sample mix was transferred on to the assembled GFX Micro spin column and collection tube. It was incubated at room temperature for 1 minute and centrifuged at 16000 x g for 30 seconds. The flow through was discarded by emptying the collection tube and placed the GFX Micro spin column back inside the collection tube. Simple binding steps were repeated until total samples volume was loaded.

3.2.9.7.3. Wash and dry

500 µl Wash buffer type 1 was added to the GFX Microspin column and then spined the assembled column and collection tube at 16000 x g for 30 seconds. Collection tube was discarded and the GFX Microspin column was transferred to a DNase free 1.5 ml micro centrifuge tube.

3.2.9.7.4. Elution

50 µl elution buffer type 4 was added to the center of the membrane in the assembled GFX Microspin column and simple collection tube then incubated at room temperature for 1 minute. Then assembled column and sample collection tube was spined at 16000 x g for 1 minute to recover the purified DNA. Purified DNA was stored at -20^{0}C for further use.

3.2.10. Cloning of DNA fragments

Cloning of DNA fragments into pGEM- T Easy vectors involved the following steps, which are described below.

3.2.10.1. Ligation of DNA fragment with pGEM- T Easy vector

The DNA fragment of *opdB*, *pfr1* and *pfr2* gene and the pGEM- T Easy vector in which it is to be cloned were digested with T4 DNA ligase enzyme to generate compatible ends for ligation. The individual DNAs were mixed so as to have a vector and insert DNAs in the ratio of 1:3 in case of sticky ends and 1:5 to 1:10 in case of blunt ends. The ligation was done (as per the Promega protocol with slight modification) in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25^0C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were vortexed, spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4^0C. The ligation mix was used directly for transformation and unused ligation mixture was stored at –20^0C for future use.

3.2.10.2. Transformation

The frozen aliquots of JM109 high efficiency competent cells was removed from -70^oC storage and placed in an ice bath thawing (for 5-10 min.). Competent cells were mixed by gentle flicking the tube. 2 µl ligation mixture and 50 µl JM109 competent cells were mixed in 1.5 ml eppendorf tube and placed the tube in ice for 20 minutes. The cells were given heat shock at 42^0C for 45 sec before snap cooling on ice for 2 minutes. Immediately thereafter 950 µl of pre warmed SOC (Appendix–III.6) was added to the cells and the suspension was incubated at 37^0C for 1 hr and 30 min. with shaking of 150 rpm in a shaking incubator. 100 µl IPTG and 20 µl X-GAL was spreaded over the surface of LB agar plates with ampicillin (50µg/ml). After incubation of transformation culture 100 µl of each transformation culture were plated onto antibiotic agar plates in duplicate and incubated at 37^0C for overnight (16-20 hr). Colonies that appeared were screened for the presence of plasmids.

3.2.10.3. Screening for recombinants

Both white and blue colonies were grown in the plate. Larger single white colonies were picked up individually and inoculated into LB broth (Appendix–III.3) containing final concentration of ampicilin (50µg/ml) and kept in an water bath cum shaker (at 37°C and 150 rpm) for 16 hrs. Subsequently, the plasmid DNA was isolated from all the bacterial cultures using the kit.

3.2.10.4. Protocol for plasmid preparation (by using illustra plasmid prep mini spin kit)

3.2.10.4.1. Harvesting of bacterial culture

1.5 ml bacterial culture was taken in eppendorf tube and centrifuged for 30 second at 16000 x g. Poured off and supernatant was discarded.

3.2.10.4.2. Lysis

175 µl lysis buffer type 7 was added and pellet was resuspended. Then 175 µl lysis buffer type 8 was added and mixed by gentle inversion till solution became clear and viscous. After 5 min of lysis reaction 350 µl lysis buffer type 9 was added and mixed by gentle inversion till percipitate was evenly dispersed. Then it was centrifuged for 4 min at 16000 x g.

3.2.10.5.3. Plasmid binding

Supernatant was transferred into plasmid mini column inside collection tube. Then it was centrifuged at 30 sec at 16000 x g and flowthrough was discarded.

3.2.10.4.4. Wash and dry

400 µl Wash buffer type 1 was added to the plasmid mini column and then spined the assembled column and collection tube at 16000 x g for 30 seconds. Flowthrough and collection tube was discarded and the plasmid mini column was transfered to a new DNase free 1.5 ml micro centrifuge tube.

3.2.10.4.5. Elution

100 µl elution buffer type 4 was added to the center of the membrane in the assembled plasmid mini column and simple collection tube then incubated at room temperature for 30 sec. Then assembled column and sample collection tube was spined at 16000 x g for 30 sec to recover the purified plasmid DNA. Purified plasmid DNA was kept at -20^{0}C for storage.

3.2.10.5. Confirmation of clones

Confirmation of clones was done by Restriction Enzyme digestion of plasmid DNAs and Colony PCR of plasmid colonies.

3.2.10.5.1. Restriction Enzyme digestion (to check the insert size)

After checking the quality of the plasmid DNAs in agarose gel electrophoresis, they were subjected to restriction enzyme digestion using *Eco*R1. In 10 µl digestion mixture 5 µl plasmid DNA, 1 µl *Eco*R1 (Promega), 1 µl *Eco*R1 buffer (Promega) and 3 µl ultrapure water was added. After 4 hrs. digestion at 37°C in water bath 2µl 6X loading dye was added to the mixture and analyzed by running 1.2% agarose gel electrophoresis alongside a DNA molecular weight marker. Release of the expected size fragment confirmed the recombinants.

3.2.10.5.2. Colony PCR

This protocol was designed to quickly screen for plasmid inserts directly from *E. coli* colonies. Colonies were screened for recombinants by colony PCR as per procedure described in Promega protocols. PCR was carried out in the presence of insert specific primers. The colony PCR reaction mixture was similar to PCR reaction mixture, only template DNA was not added. To each PCR tube containing the PCR reaction, a single colony was added. For each amplification reaction white colonies were added in two PCR tubes and blue colonies were added in two tubes. A fine yellow pipette tip attached to a pipetter was used to separate a colony from culture and pipetted up and down to mix the colony into PCR reaction mixture (The amount of cells were small, just a touch was done, the small amount required to fill the end of the opening was sufficient). Sufficient mixing of the colony in PCR tube was done for complete cell lysis and high yields. The conditions of amplification applied were similar to those applied for the amplification of the specific genes. The amplified products were analyzed by agarose gel electrophoresis using standard molecular size markers.

3.2.11. Sequencing

Purified plasmids of *opdB*, *pfr1* and *pfr2* genes were sequenced from Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore. The sequence obtained was then matched using BLAST (Biological Local Alignment Search Tool) software. After confirmation of the *opdB*, *pfr1* and *pfr2* genes nucleotide sequences of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the nucleotide sequences were submitted to GenBank, NCBI database.

3.2.11.1. Sequence analysis

After getting the accession numbers of individual gene sequences Phylogenetic and sequence analysis of the *opdB*, *pfr1* and *pfr2* genes of *T.evansi* was done. The phylogenetic and sequence analysis was done by the use of Clustal X and MEGA5 softwares. Phylogenetic tree analysis of *opdB*, *pfr1*and *pfr2* genes was done by using Neighbor-Joining (NJ) method and maximum parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

3.2.11.2. Multiple sequence alignments

The amino acid sequences of the *opdB*, *pfr1* and *pfr2* genes from *T. evansi* was BLASTed against similar sequences in the public database (http://www.ncbi.nlm.nih.gov/). The sequences of opdB proteins of *T. brucei* (GenBank Accession No. AF078916 and XM824253), *T. evansi* (GenBank Accession No. AY546084), *T. cruzi* (GenBank Accession No. XM804874 and U69897), *L. amazonensis* (GenBank Accession No. EF392367), *L. donovani* (GenBank Accession No. GQ491028), *L. infantum* (GenBank Accession No. XM001463502) and *L. major* (GenBank Accession No. AF109875) were collected and sequence homology between species determined. For sequence homology determination of pfr1 protein the sequences of *T. brucei* (GenBank Accession No. XM838928 and Z25827), *T. evansi* (GenBank Accession No. EU366960 and FJ968743), *T. cruzi* (GenBank Accession No. XM804737 and AF005195), *L. infantum* (GenBank Accession No. AY702344 and XM003392645) and *L. major* (GenBank

Accession No. XM003722211) and for pfr2 protein the sequences of *T. brucei* (GenBank Accession No. XM842234, X14819 and L30155), *T. evansi* (GenBank Accession No. GQ392136, EU258755 and FJ901341) and *T. cruzi* (GenBank Accession No. M97548, XM809076 and FJ222461) were collected. Multiple sequence alignments of obtained protein sequences of *opdB*, *pfr1* and *pfr2* genes were performed with Clustal W program version 2.1 at EBI (expasy proteomics tools) with default parameters.

3.2.11.3. Three dimensional structure model of opdB, pfr1 and pfr2 proteins

3.2.11.3.1. Building three dimensional structure model of opdB, pfr1 and pfr2 proteins using computational approach

The 3D model of opdB, pfr1 and pfr2 were built by homology modeling based on high-resolution crystal structures of homologous proteins. A basic alignment search tool (BLAST, Altschul *et al*., 1990) search was performed for selecting the 3D models of the closest homologues available in the Brookhaven Protein Data Bank (PDB).The gene sequences of *opdB*, *pfr1* and *pfr2* were obtained from National Center for Biotechnology (NCBI) GenBank database. The 2XE4 showed a high level of sequence identity with opdB, 3LP5 with pfr1 and 2LVI with pfr2. The coordinates of crystal structure of 2XE4, 3LP5 and 2LVI were used as template to build the initial models of opdB, pfr1 and pfr2. The 3D model of opdB, pfr1 and pfr2 were generated by the automated homology modeling software MODELLER9v6 (http://salilab.org) on windows operating environment (Sali & Blundell, 1993). This programme is used for comparative protein structure prediction that optimally satisfies spatial restraints. The spatial restraints are expressed as probability density functions (pdfs) for the features restrained. The pdfs restrain C^{α}- C^{α} distances, main-chain N-O distances, main – chain and side – chain dihedral angles. The 3D model of the protein was obtained by optimization of the molecular pdf such that the model violates the input restraints as little as possible.

3.2.11.3.2. Energy Minimization

The models thus generated were screened for unfavorable steric contacts and remodeled using Discovery studio software (license obtained from www.accelrys.com) or manual rotations. Hydrogen atoms were added explicitly to the proteins, through the Biopolymer module and they were subjected to energy minimization using steepest discent and conjugate gradient, respectively.

3.2.11.3.3. Evaluation of Modeled Structures

The protein models of opdB, pfr1 and pfr2 generated by comparative modeling and energy minimization were checked for stereochemical quality, regions of unusual geometry and overall structure assessment via the tool PROCHECKv3.4.4. The Ramachandran Plots generated were studied for the same and the G-factors were observed. RMSD (Cg) of the modeled proteins, with respect to the best template (2XE4, 3LP5 and 2LVI) were measured by pairwise structure alignment using the Combinatorial Extension algorithm (CE), available at the San Diego Supercomputer Center (http://cl.sdsc.edu).

4. Results

Trypanosomosis is a very serious and often fatal blood protozoan disease of camels and has been observed in domestic and wild animals and recent reports suggest its ability to infect humans with widest geographical range. Chemotherapeutic,

chemoprophylactic and fly control strategies are not quite enough to control this disease. Recent effort towards the development of a vaccine against *Trypanosoma evansi* has identified several promising candidate vaccine antigens, including non-varient genes of this parasite. Keeping this in view, the present study was undertaken to amplify the *opdB*, *pfr1* and *pfr2* genes of *T. evansi* from camels by Polymerase Chain Reaction (PCR), clone the amplicons in a suitable plasmid vector and sequence the genes as these candidate genes could be helpful in future for developing vaccines against the organism. The results obtained are as follows:

4.1. Identification of Camel (*Camelus dromedarius*) infected with 'surra' (*Trypanosoma evansi*) infection

Initially, the camel suffering from 'surra' disease was identified by its symptomatic characteristics and the infected host with *T. evansi* has been shown in Plate 2.

4.2. Stained blood film preparation of *T. evansi*

A Giemsa stained blood smear of *T. evansi* collected from the infected camel was prepared to confirm the infection and has been presented in Plate 3.

4.3. Propagation of *T. evansi* in mice and its purification

The *T. evansi* collected from camel blood were propagated in mice (Giemsa stained blood smear of mice blood shown in Plate 4) and were then purified employing DEAE-cellulose chromatography method and were stained using Giemsa stain as shown in Plate 5.

4.4. Isolation and qualitative confirmation of genomic DNA

The total genomic DNA was isolated from the pellets of *T. evansi* using Proteinase K digestion and subsequent phenol-chloroform extraction method as mentioned in materials and methods. The genomic DNA was analyzed in 0.8% analytical agarose gels and was found to be intact without much smearing and have been presented in Plate 6.

4.5. Extraction and qualitative confirmation of total RNA

After extraction of total RNA the extracted eluent was then subjected to agarose gel electrophoresis to confirm the presence of RNA. Two distinct bands on the plate could be visualized under UV illuminator which were confirmed as 18s RNA and 28s RNA and have been presented in Plate 7.

4.6. c-DNA synthesis and purification

After c-DNA synthesis and its purification the agarose gel electrophoresis technique was employed once again here for qualitative confirmation of c-DNA. A smear like pattern was observed under UV illuminator which confirmed the presence of c-DNA and has been presented in Plate 8.

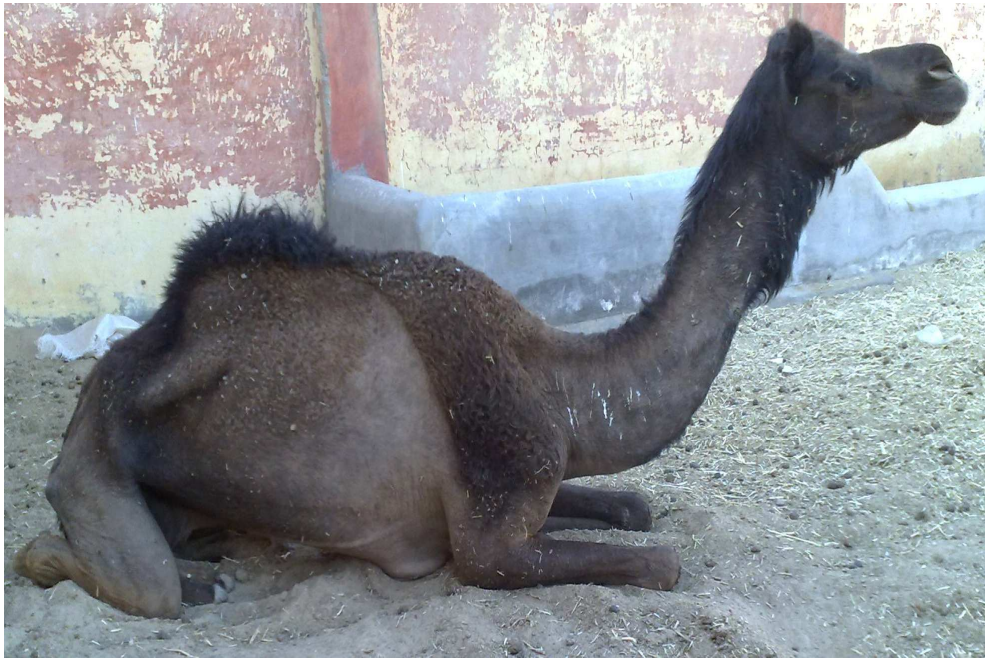


Plate 2. Camel infected with ‘surra’ disease

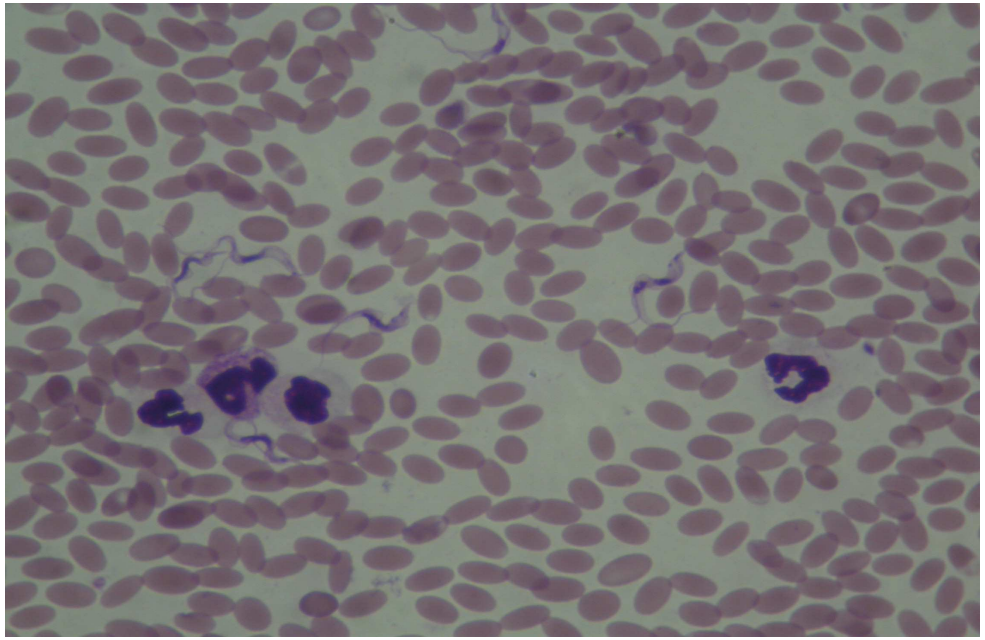


Plate 3. Giemsa stained blood smear of *T. evansi* from camel

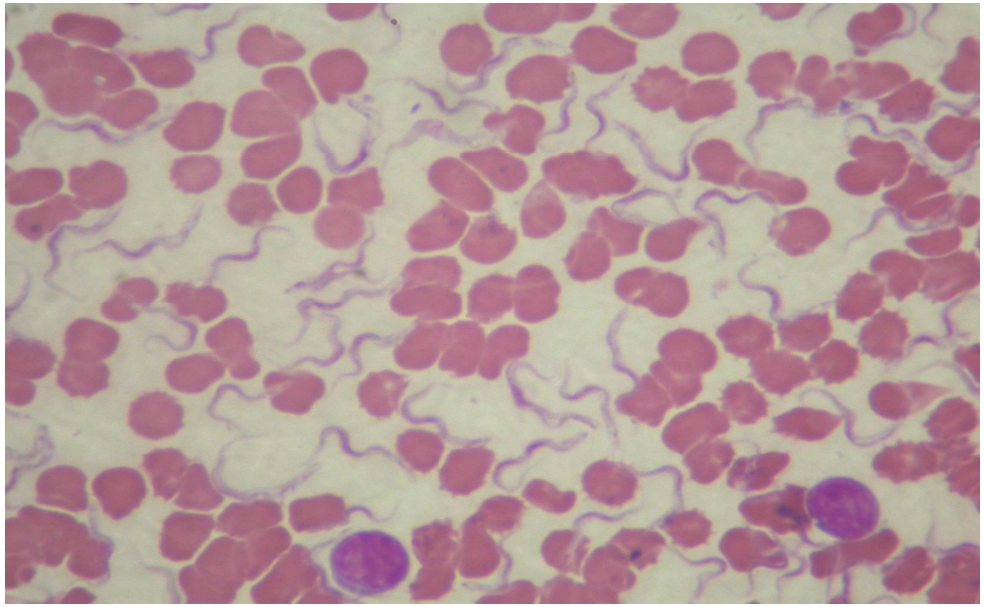


Plate 4.Giemsa stained blood smear of *T. evansi* from mice

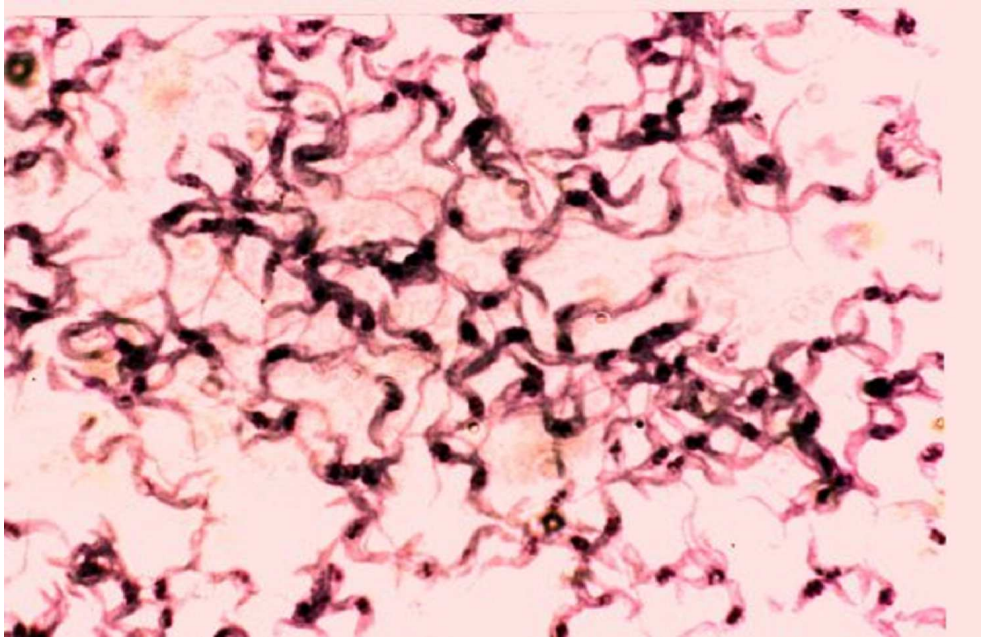


Plate 5. Purified and Giemsa stained *T. evansi*

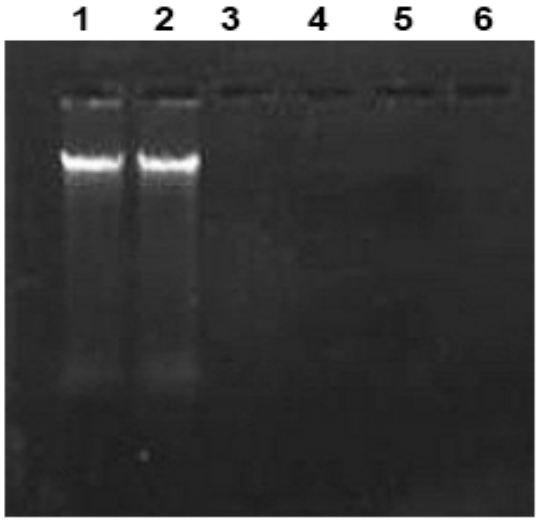


Plate 6. Genomic DNA of *Trypanosoma evansi* in lane 1 and 2

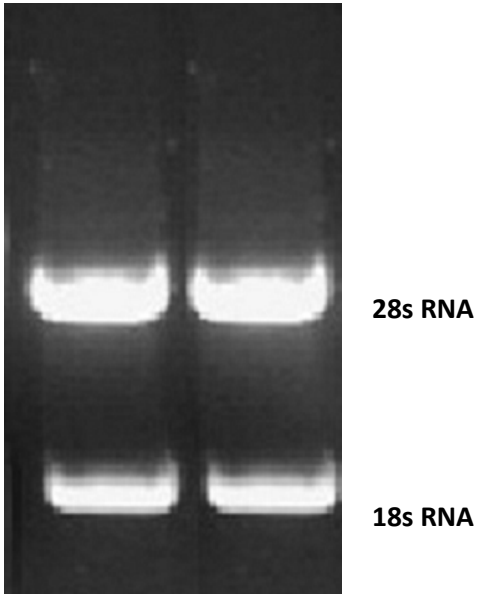


Plate 7. 18s & 28s RNA visualized under UV Trans illuminator

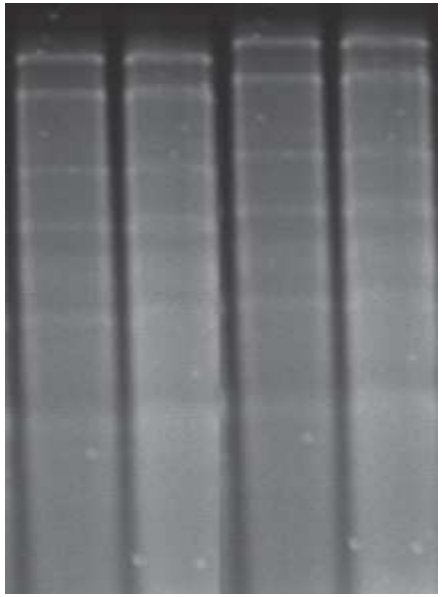


Plate 8. A smear like pattern of c-DNA visualized under UV Trans illuminator

4.7. Amplification of Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *T. evansi* by PCR

Total genomic DNA was isolated from the pellets of *T. evansi* and used as template for amplification by PCR. Gene specific forward and reverse primers were used for amplification and the amplicons analyzed by agarose gel electrophoresis as per protocol

mentioned in the materials and methods. To confirm the size of amplicons, the sample (amplicon) was run on gel electrophoresis using molecular weight marker (1 kbs plus DNA ladder). The amplification band of *opdB* gene was obtained in between 3000 bp and 2000 bp which has been presented in Plate 9. The amplification band of *pfr1* and *pfr2* gene was obtained in between 2000 bp and 1650 bp which has been presented in Plates 10 and 11.

4.8. Amplification of the Paraflagellar rod 1 and Paraflagellar Rod 2 gene of *T. evansi* by RT- PCR (Reverse Transcription PCR)

From the c-DNA obtained, the desired amplicons (both *pfr1* and *pfr2* genes) were then amplified using gene specific primers. To confirm whether amplification took place or not, the samples (amplicons) were run on gel electrophoresis using molecular weight marker (1 kbs plus DNA ladder). The amplification band was obtained in between 2000 bp and 1650 bp which has been presented in Plate 12 and 13.

4.9. Cloning of Oligopeptidase B, Paraflagellar Rod 1 and Paraflagellar Rod 2 genes of *T. evansi* into pGEM-T Easy vector

The amplicons (obtained from genomic DNA amplification) were purified from the LMP agarose gel and ligated with pGEM-T Easy vector (Promega). 100 µl of transformation culture was plated onto X-gal-IPTG-Ampicillin agar plate. There were several white colonies along with a few blue colonies (plate no. 14). The blue colonies represent the presence of vector alone but few blue colonies may contain vector with insert. The white colonies may represent recombinant clones carrying insert in the plasmid. The white colonies were screened for the presence of vector with insert.

4.9.1. Confirmation of clones by restriction digestion

Plasmid DNAs were extracted from positive colonies grown in LB medium containing ampicillin, digested with *Eco*RI and analyzed by 1.2% analytical agarose gel electrophoresis using 1kb plus molecular weight marker. Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon digestion with *Eco*RI (lanes 2 & 3), the less intense lower band may correspond to the insert. Release of DNA fragments of around 2092 bp for *opdB*, 1769 bp for *pfr1* and 1767 bp for *pfr2* gene was found after restriction enzyme digestion, the results of which have been presented in Plate 15 to 17.

4.9.2. Confirmation of clones by Colony PCR

Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and results were analyzed by agarose gel electrophoresis using 1kb plus molecular weight marker. For *opdB* and *pfr2* gene amplifications were found in wells of white colonies but for *pfr1* gene amplification was also found in one blue colony (Plate 18 to 20).

4.10. Sequencing

After confirmation of clones of *opdB*, *pfr1* and *pfr2* genes the plasmid DNAs along with their respective forward and reverse primers were sent to Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore, for getting the sequences. The coding sequences of *opdB*, *pfr1* and *pfr2* genes according to the results obtained were of 2092 bp, 1769 bp and 1767 bp, respectively. These sequences were then matched using BLAST software. After confirmation of the *opdB*, *pfr1* and *pfr2* genes nucleotide sequences of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the sequences were submitted to GenBank, NCBI database to which the assigned accession numbers are JQ909240 for *opdB* (Plate 21), JQ909241 for *pfr1* (Plate 25) and JX020770 for *pfr2* gene (Plate 29).

4.10.1. Sequence analysis

Sequence analysis revealed that the length of the coding sequences of *opdB*, *pfr1* and *pfr2* genes of *T. evansi* from Bikaner, India were 2092 bp, 1769 bp and 1767 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology is based on the Neighbor-Joining (NJ) method with100% bootstrap values. The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic tree analysis of *opdB*, *pfr1*and *pfr2* gene using maximum parsimony (MP) also showed same topology as NJ method. The results, along with the assigned accession numbers and the names of workers who submitted them are presented in Plate 22a, 22b, 26a, 26b, 30a and 30b and Tables 4.1 to 4.6.

4.10.2. Multiple sequence alignment

Multiple sequence alignment of obtained protein sequences of *opdB*, *pfr1* and *pfr2* genes was performed with Clustal W at EBI (expasy proteomics tools). The results of amino acid alignments (Clustal 2.1 multiple sequence alignment) are presented in Plate 23, 27, and 31. The asterisks below the alignments indicate the positions of highly conserved amino acid residues.

Obtained opdB protein sequence of *T. evansi* was of 697 amino acids (GenBank Accession No. JQ909240) which showed 100% amino acid sequence identity of *T. evansi*, GenBank Accession No. AY546084, 99% of *T. brucei*, GenBank Accession No. AF078916, 98% of *T. brucei*, GenBank Accession No. XM824253, 71% of *T. cruzi* (GenBank Accession No. XM804874 and U69897), 61% of *L. amazonensis* (GenBank Accession No. EF392367), *L. donovani* (GenBank Accession No. GQ491028) and *L. infantum* (GenBank Accession No. XM001463502) and 60% of *L. major* (GenBank Accession No. AF109875).

Obtained pfr1 protein sequence of *T. evansi* was of 589 amino acids (GenBank Accession No. JQ909241) which showed 100% amino acid sequence identity of *T. evansi*, GenBank Accession No. FJ968743 and *T. brucei*, GenBank Accession No. XM838928, 99% of *T. evansi*, GenBank Accession No. EU366960, 93% of *T. brucei* (GenBank Accession No. Z25827) and *T. cruzi* (GenBank Accession No. XM804737 and AF005195), 84% of *L. infantum* (GenBank Accession No. AY702344), 85% of and *L. major* (GenBank Accession No. XM003722211) and *L. infantum* (GenBank Accession No. XM003392645).

Obtained pfr2 protein sequence of *T. evansi* was of 589 amino acids (GenBank Accession No. JQ909241) which showed 99% amino acid sequence identity of *T. evansi* (GenBank Accession No. GQ392136, EU258755 and FJ901341) and *T. brucei* (GenBank Accession No. XM842234 and X14819), 98% of *T. brucei* (GenBank Accession No. L30155) and 90% of *T. cruzi* (GenBank Accession No. (GenBank Accession No. M97548, XM809076 and FJ222461).

4.10.3. 3D structure of obtained opdB, pfr1 and pfr2 proteins

The structures of opdB, pfr1 and pfr2 protein have been determined by using homology modeling protocol. BLASTP search was performed against PDB with default parameters to find suitable templates for homology modeling. Based on the maximum identity with high score and lower e-value 2XE4, 3LP5 and 2LVI were used as the template for homology modeling. 3D models of opdB, pfr1 and pfr2 were created using Modeller9v6. The input given was sequence alignment in PIR format, generated from ClustalW and the 3D structure files of the templates. The script used to generate the models was model-multiple.py. Sequence identity was 52%, 33% and 32% between opdB & 2XE4, pfr1 & 3LP5 and pfr2 & 2LVI, respectively. In the following steps 2XE4, 3LP5 and 2LVI were chosen as reference structure for modeling opdB, pfr1and pfr2. Coordinates from the reference protein (2XE4, 3LP5 and 2LVI) to the structurally Conserved Regions (SCRs), structurally variable region (SVRs), N-termini and C-termini were assigned to the target sequence based on the satisfaction of spatial restraints. All side chains of the model protein were set by

rotamers. Of the 20 structures generated for the same target (opdB, pfr1 and pfr2) and the template (2XE4, 3LP5, 2LVI), the one with the lowest value of the MODELLER objective function was selected as the best model for opdB, pfr1 and pfr2. The models thus generated were subjected to energy minimization and subsequent structure validation. The final 3D structure of opdB are presented in Plate 24a and 24b, pfr1 in Plate 28a and 28b and pfr2 in Plate 32a and 32b. The alpha helix and beta sheets are shown as helices and ribbons, respectively. The figure was prepared by PyMol.

The final stable structure of opdB have 9 sheets, 4 beta alphabeta unit, 23 beta hairpins, 11 beta bulges, 39 strands, 22 helices, 20 helix-helix interfaces, 72 beta turns and 6 gamma turns. Similarly, the stable structure of pfr1 have 4 sheets, 1 beta-alpha-beta unit, 2 beta hairpins, 2 beta bulges, 9 strands, 35 helices, 57 helix-helix interfaces, 105 beta turns and 16 gamma turns and pfr2 have 4 helices, 4 helix-helix interact, 3 beta turns and 2 gamma turns.

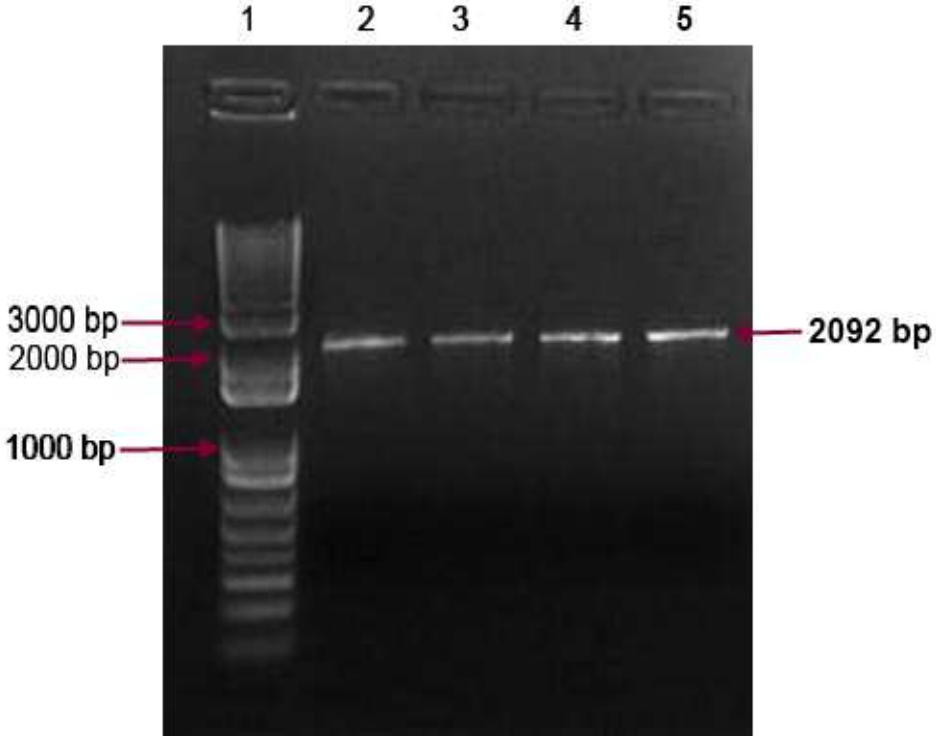


Plate 9. Amplification of Oligopeptidase B gene of *T. evansi* by PCR

1. 1Kb plus DNA Ladder

2 – 5. Amplicons

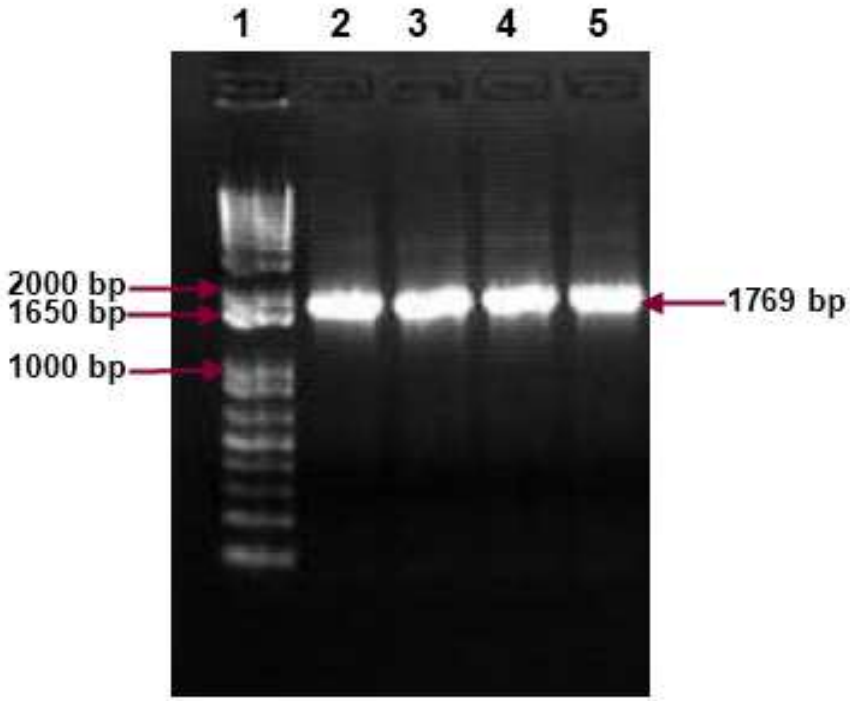


Plate 10. Amplification of Paraflagellar rod 1 gene of *T. evansi* by PCR

1. 1Kb plus DNA Ladder 2 – 5. Amplicons

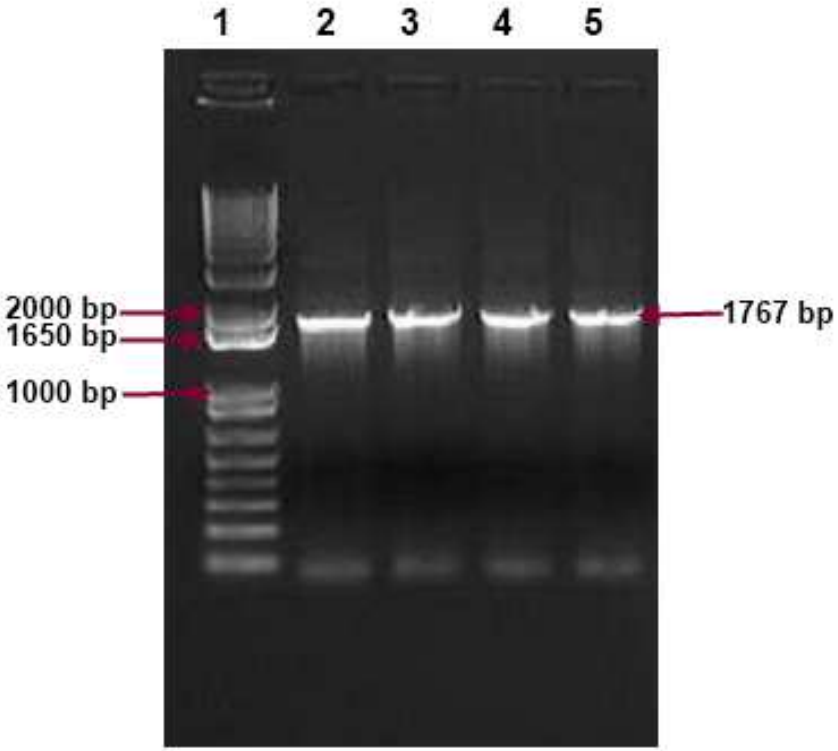


Plate 11. Amplification of Paraflagellar rod 2 gene of *T. evansi* by PCR

1. 1Kb plus DNA Ladder
2 – 5. Amplicons

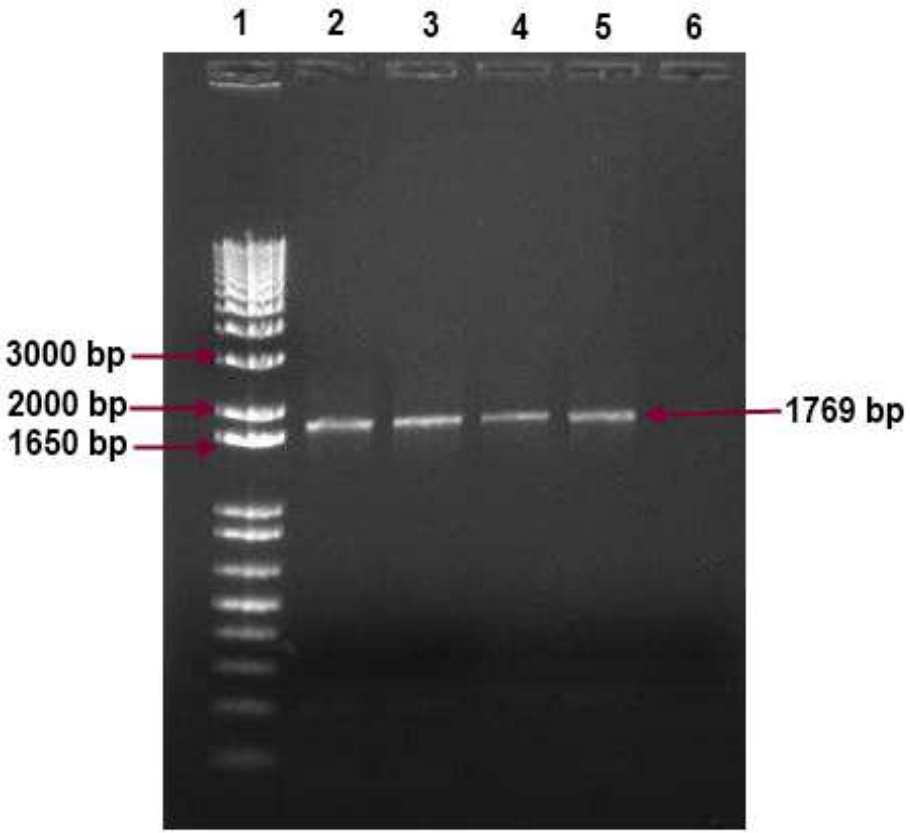


Plate 12. Amplification of Paraflagellar rod 1 gene of *T. evansi* by RT-PCR

1. 1Kb plus DNA Ladder 2 – 5. Amplicons
6. Control

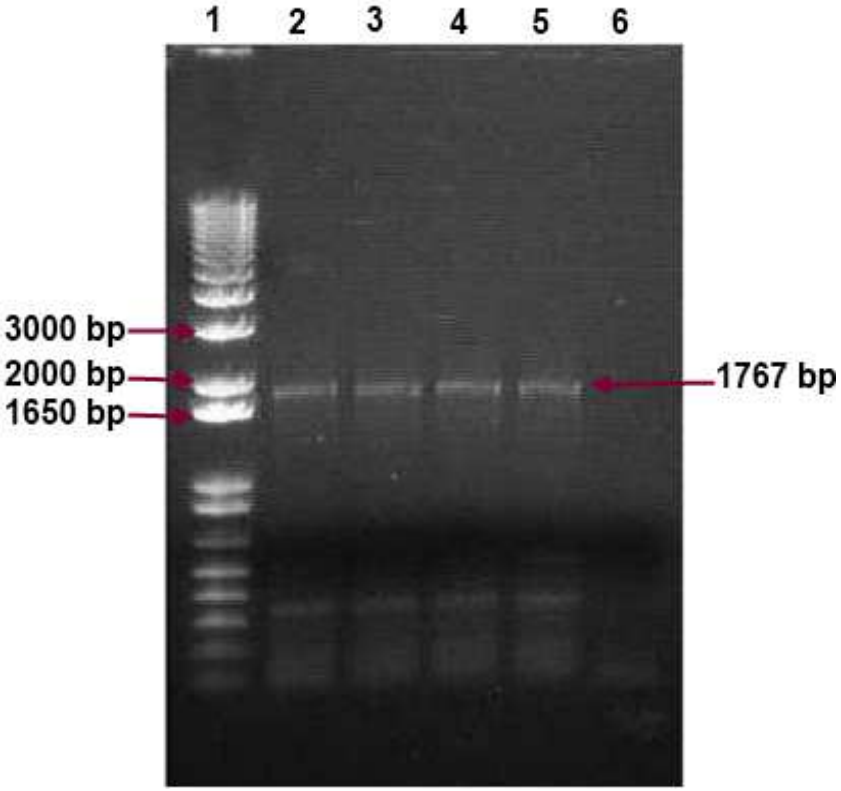


Plate 13. Amplification of Paraflagellar rod 2 gene of *T. evansi* by RT-PCR

1. 1Kb plus DNA Ladder 2 – 5. Amplicons
6. Control

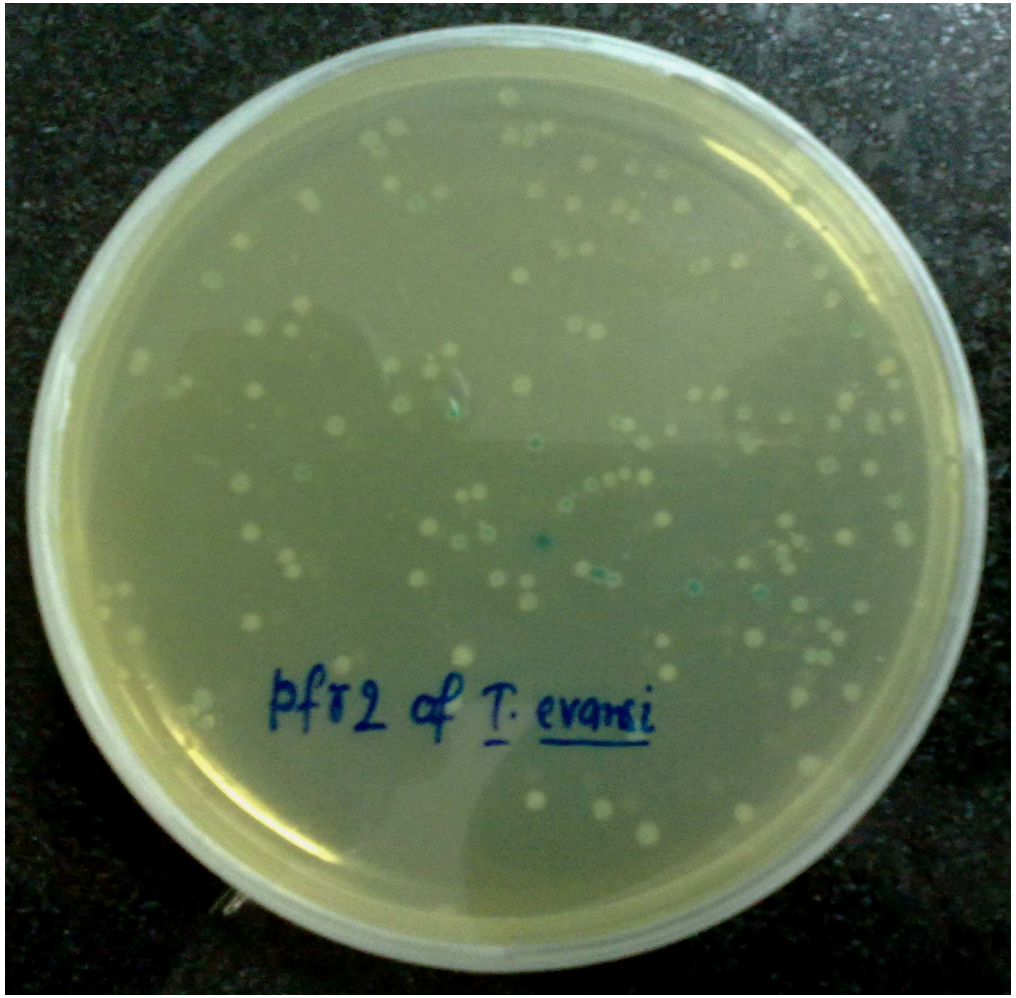


Plate 14. Appearance of Blue and white colony in LB agar plate showing culture of Paraflagellar rod 2 gene of *T.*

evansi

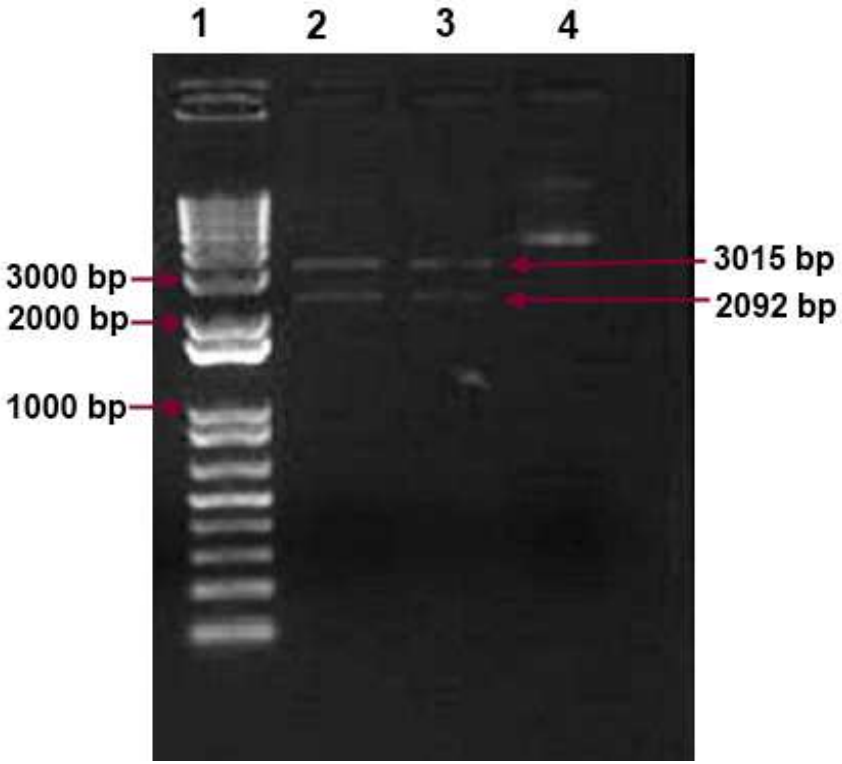


Plate 15. Oligopeptidase B gene fragments of *T. evansi* after restriction digestion of *opdB* gene plasmid

Legends

1. 1Kb plus DNA Ladder

2 – 3. Oligopeptidase B gene clone

4. Uncut plasmid

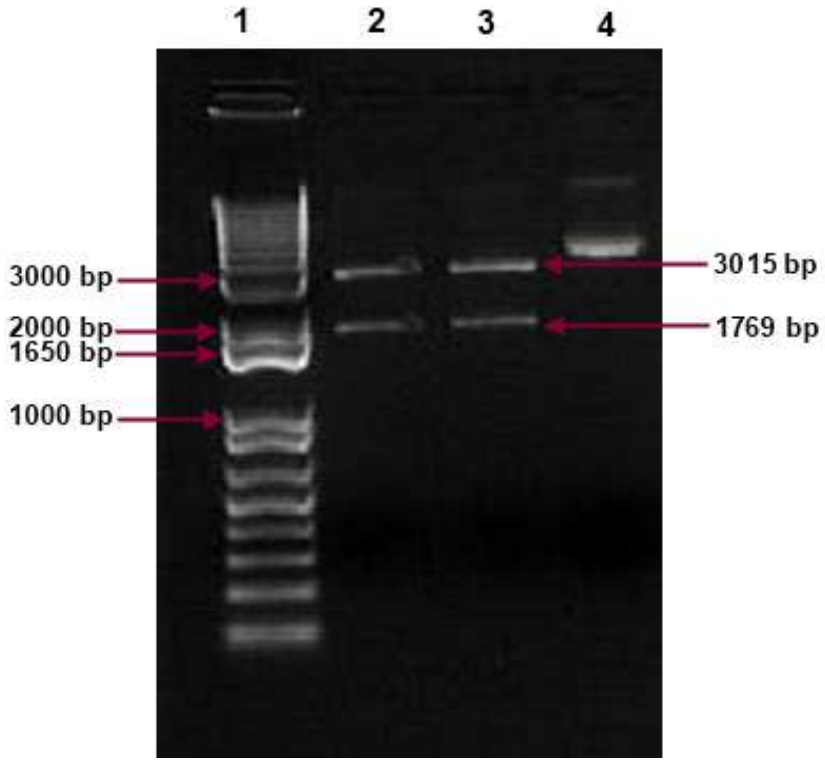


Plate 16. Paraflagellar rod 1 gene fragments of *T. evansi* after restriction digestion of *pfr1* gene plasmid

Legends

1. 1Kb plus DNA Ladder

2 – 3. Paraflagellar Rod 1 gene clone

4. Uncut plasmid

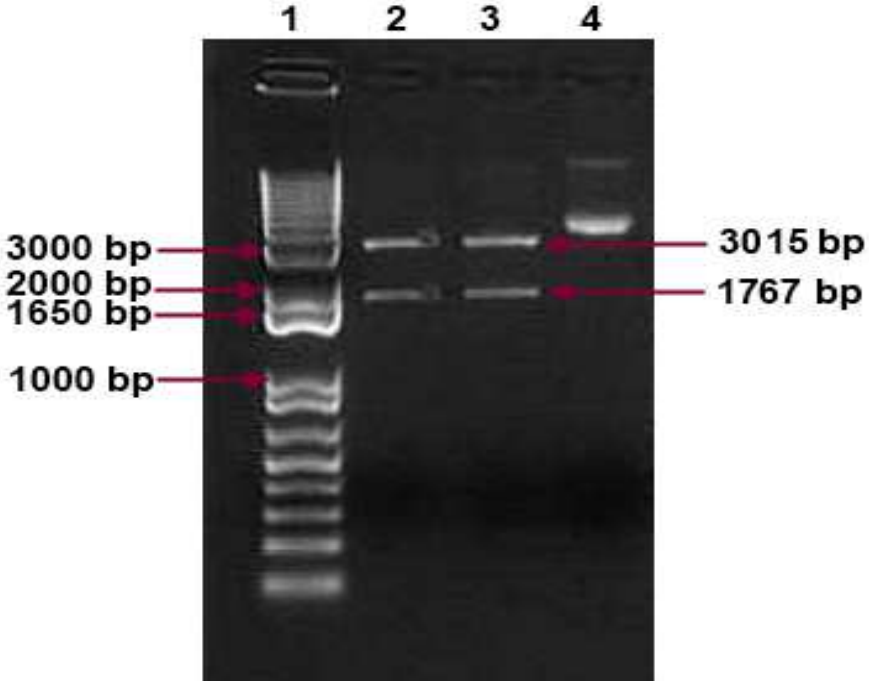


Plate 17. Paraflagellar rod 2 gene fragments of *T. evansi* after restriction digestion of *pfr2* gene plasmid

Legends

1. 1Kb plus DNA Ladder

2 – 3. Paraflagellar Rod 2 gene clone

4. Uncut plasmid

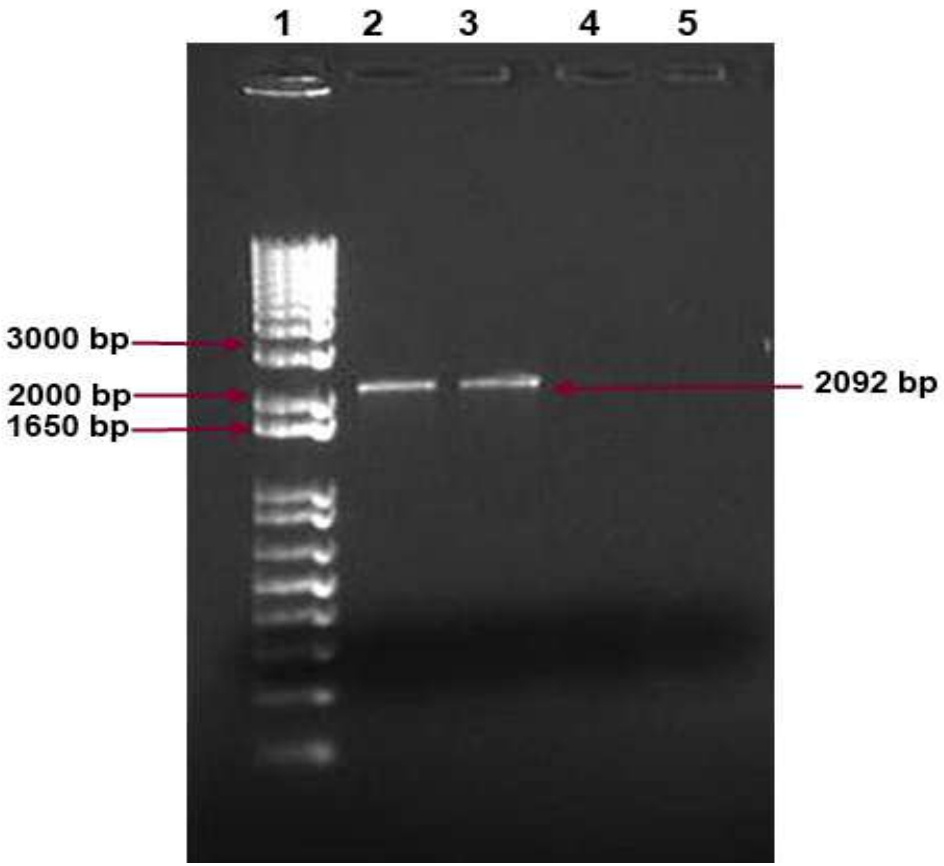


Plate 18. Amplification of Oligopeptidase B gene of *T. evansi* by Colony-PCR

1. 1Kb plus DNA Ladder 2 – 3. PCR reaction with white colony shows

Amplification

4 – 5. PCR reaction with blue colony shows absence of Amplification

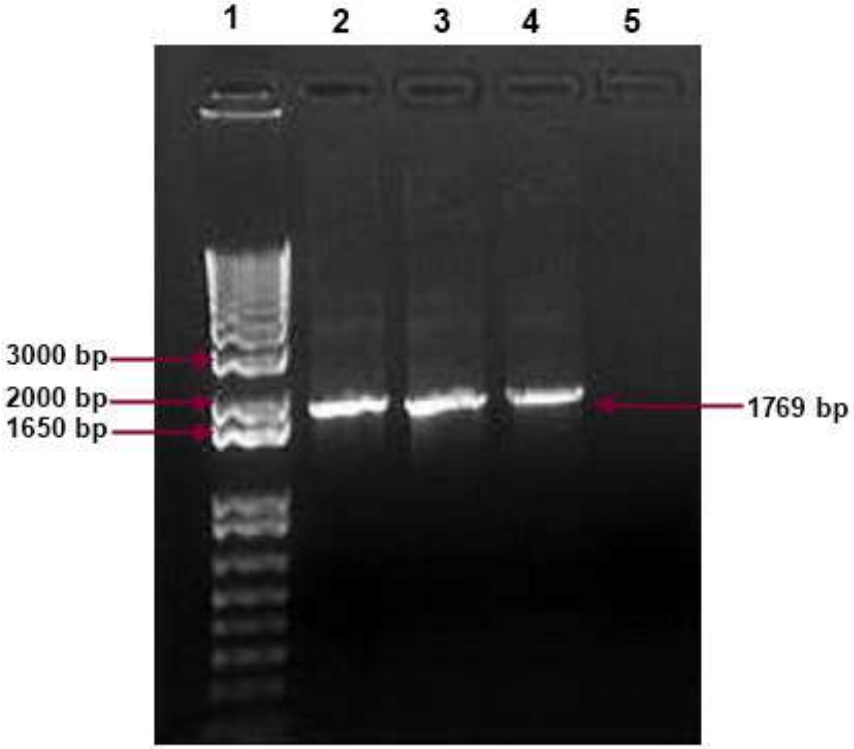


Plate 19. Amplification of Paraflagellar rod 1 gene of *T. evansi* by Colony-PCR

1. 1Kb plus DNA Ladder

2 – 3. PCR reaction with white colony shows Amplification

4. PCR reaction with blue colony shows

Amplification

5. **PCR reaction with blue colony shows absence of Amplification**

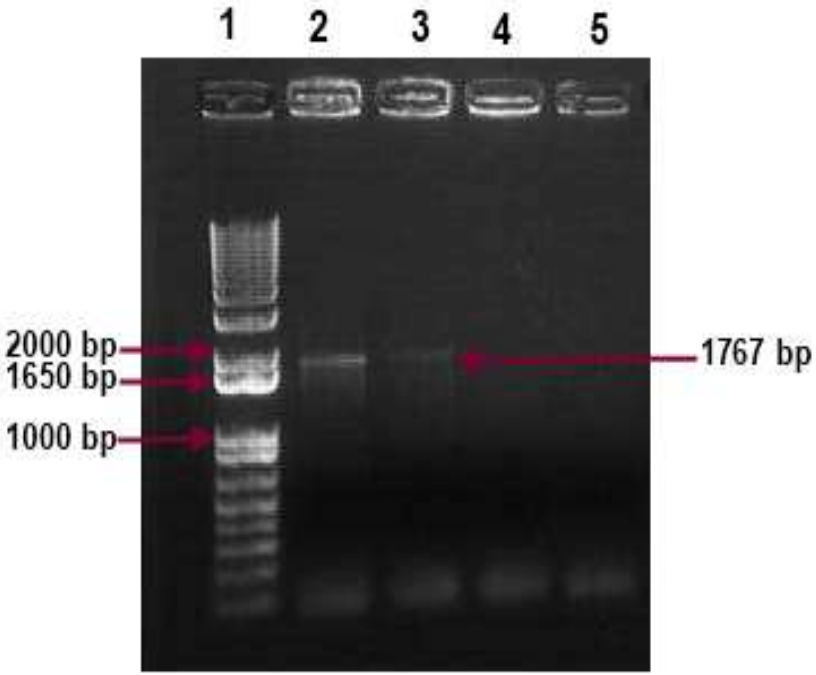


Plate 20. Amplification of Paraflagellar rod 2 gene of *T. evansi* by Colony-PCR

1. 1Kb plus DNA Ladder

2 – 3. PCR reaction with white colony shows Amplification

4 – 5. PCR reaction with blue colony shows absence of Amplification

ORIGIN

```
  1 ATGCAAACTG AACGTGGTCC AATCGCCGCA CATCGGCCCC ACGAGGTGGT TTTTGGCAAA
 61 GTTGAGGGCT TAGACCGCGG CGCCAACCCA ATGGACCCGC CCCGCCGCAG GGTCGACCCG
121 CTCTTTTGGC TTCGCGATGA TAACCGTGCC GATCCCGAAG TCCTCGCCCA CCTCCACTTG
181 GAGAAGGATT ATTACGAGAA GCGCGCGGTC GATATTAAAG ACCTGGCGGA AACTATTTAC
241 CAAGAGCACA TTTCACACAT TGAAGAAACG GACATGTCGG CCCCCTACGT CTACGACCGC
301 TTCTTGTACT ACACGCGTGA TGTAAAGGGG TTGTCGTACA AGCTTCATTG TCGCGTACCG
361 GCCGGGAAGA CGCCGGGTGA GGGTGAGGAT GAGGAGATTG TACTGGACGA AAATAAGCTC
421 GCGGAGGGCA AATCTTTTTG TGTCGTGGGT TGCGTGGCGC CCGCCCCACC AGAACATGCG
481 CTTGTAGCAT ACTCCGTTGA TTACTGCGGG GATGAGGTGT ACAGCATCCG TTTCGTACGG
541 GACGTGGTGG CGGACAAGGT TGAAGGCACA AACGGAAGTG TTGTGTGGGG GCCCAATGCC
601 GAATGCTTCT TTTACATTAC GAAAGATGCC TCCAAGCGTG ACAACAAGGT ATGGCGTCAC
661 ATTATTGGCC AACCGCAAAG TGAAGATGTA TGCCTCTACA CCGACGACGA TCCACTCTTC
721 TCGGTGGGTG TGGGGAGGTC GGGTGACGGC AAGACGCTGA TCATTTGCTC TATGTCATCA
781 GAAACTTCGG AGTCACATCT GTTGGATCTG CGCAAGGGGG TTAAGCACAA CACACTCGAG
```

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 841 ATGGTACGAC CCCGTGAGAA GGGGGTTCGC TACACTGTGG AGATGCACGG CACGGACACA
 901 CTGATAGTGC TGACAAATAA AGACAAGTGC GTGAATGGTA AGGTTGTATT GACCAAGCGG
 961 AGTGCACCCA CAGATTGGGG GACCGTATTA ATACCCCATG ACGACAAGGT AACTATTGAT
1021 GATGTCGTCG TATTCGCCAA ATTTGCAGTT CTATCCGGCC GCCGCGATGG TTTGACGCGC
1081 GTATGGACGG TCAGGCTCGG GCCTGACAAC CTCTTCAGCT CTGCAACGCT GAAGGAGCTG
1141 CACTTCGATG AGCCTGTTTT CACTGCCCAT GTGGTTTGTT CTCAAATGAA GACATACGAT
1201 GCGTCACTGC TGCGCCTGAG GTATTCATCC ATGACAACCC CCACTGTATG GTACGACGAG
1261 GACGTACTGA GCGGAGAACG CAAGGTTGTG AAGGCGCGTA AGGTGGGGGG CGGCTTTGAA
1321 TCGAAGAATT ACGTTTGTCG GAGGGAGTTG GCTACAGCTC CTGACGGGAC AAAAGTTCCC
1381 ATCTCGCTCG TATACGATAC CAGTATCGAT TTGAAGAAGC CCAACCCCAC CATGCTCTAT
1441 GGATACGGTT CCTACGGCAT CTGCATTGAG CCTGAGTTTA ACTCACGGTT CCTGCCGTAT
1501 GTTGATCGGG GTATGATATA TGCTATTGCG CACGTGCGAG GCGGCGGTGA GATGGGTCGT
1561 ACGTGGTATG AAGTTGGGGG AAAGTACTTG ACCAAACGGA ATACCTTCAT GGACTTTATT
1621 GCATGCGCGG AGCACCTTAT TTCCTCTGGT CTCACAACAC CCGCGCAGCT TTCTTGCGAG
1681 GGAAGAAGTG CTGGTGGATT GCTGGTGGGT GCCGTGTTGA ATATGCGGCC AGATTTATTC
1741 CACGTCGCCC TCGCGGGTGT CCCTTTTGTG GACGTAATGA CAACCATGTG TGATCCAAGC
1801 ATTCCTCTTA CGACAGGCGA GTGGGAGGAG TGGGGGAATC CGAATGAGTA CAAGTTTTTT
1861 GACTACATGA ACAGCTACAG TCCAATTGAC AACGCGCGTG CACAGGATTA CCCTCATTTG
1921 ATGATTCAGG CCGGATTGCA CGACCCACGC GTCGCGTATT GGGAACCGGC GAAATGGGCG
1981 TCAAAGCTCC GTGAACTCAA GACAGACAGC AATGAGGTGT TGCTGAAAAT GGATCTGGAG
2041 AGTGGACACT TTTCTGCAAG TGACCGCTAC AAGTACTTGC GAGAAAACGC AA
```

Plate 21. The coding sequence of Oligopeptidase B gene of *T. evansi* isolated from *Camelus dromedarius*

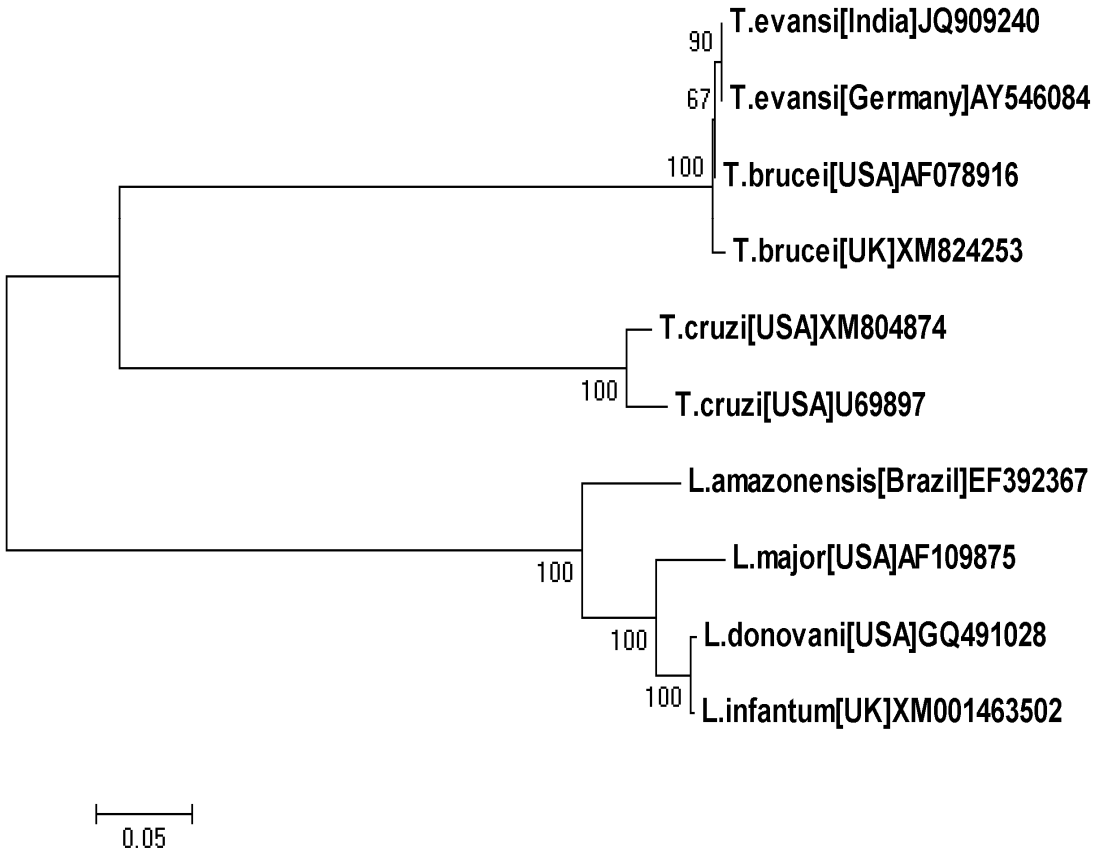

Plate 22a. Phylogenetic tree analysis of Oligopeptidase B gene using the Neighbor-Joining method

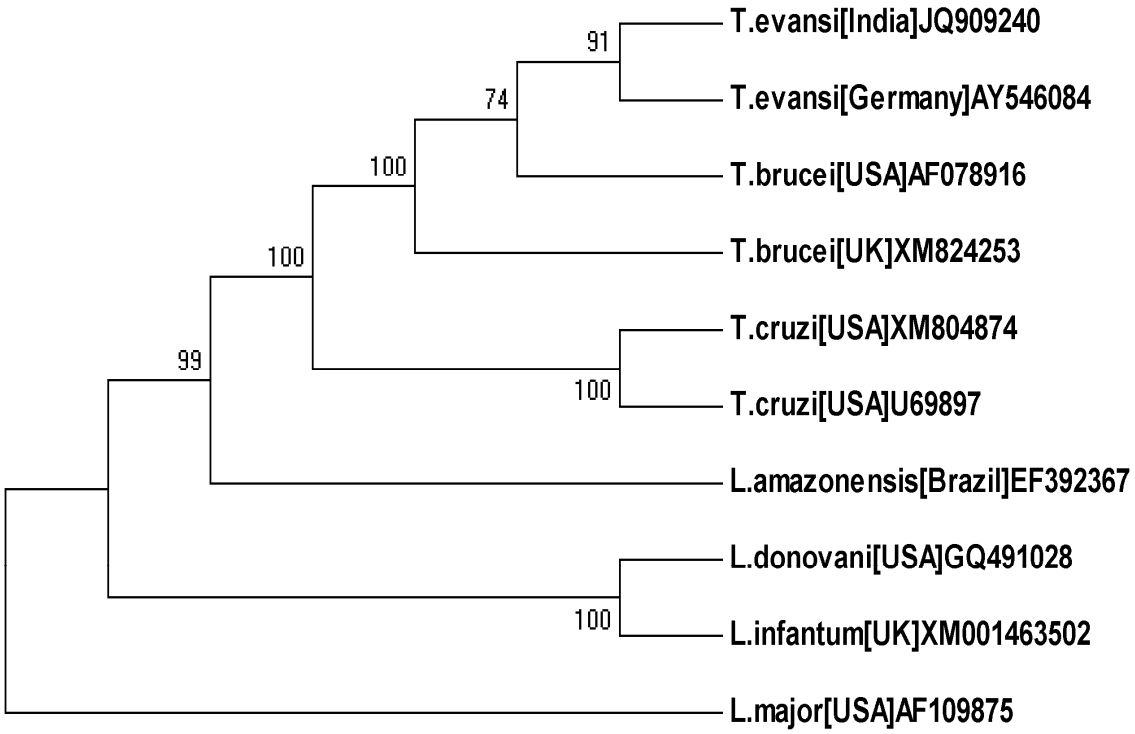

Plate 22b. Phylogenetic tree analysis of Oligopeptidase B gene using maximum parsimony method

Table 4.1: Sequence identity of Oligopeptidase B gene of *T. evansi* with other allied species

Identity of *opdB* gene for *Trypanosoma evansi*	*T.evansi* [India] JQ909240	*T.brucei* [USA] AF078916	*T.evansi* [Germany] AY546084	*T.brucei* [UK] XM824253	*T.cruzi* [USA] XM804874	*T.cruzi* [USA] U69897	*L.amazonensis* [Brazil] EF392367	*L.donovani* [USA] GQ491028	*L.infantum* [UK] XM001463502	*L.major* [USA] AF109875
T.evansi [India] JQ909240	**	99.6	100	99.0	75.7	68.4	77.6	76.2	75.9	76.2
T.brucei [USA]	99.6	**	99.6	99.2	75.8	75.1	77.9	82.7	76.2	76.4

AF078916										
T.evansi [Germany] AY546084	100	99.6	**	99.0	75.6	75.0	77.6	76.2	75.9	76.2
T.brucei [UK] XM824253	99.0	99.2	99.0	**	75.7	75.1	77.9	76.4	76.2	76.4
T.cruzi [USA] XM804874	75.7	75.8	75.6	75.7	**	98.3	72.2	72.2	72.1	75.0
T.cruzi [USA] U69897	68.4	75.1	75.0	75.1	98.3	**	74.6	74.5	74.7	73.6
L.amazonensis [Brazil] EF392367	77.6	77.9	77.6	77.9	72.2	74.6	**	93.9	93.8	92.9
L.donovani [USA] GQ491028	76.2	82.7	76.2	76.4	72.2	74.5	93.9	**	99.8	96.3
L.infantum [UK] XM001463502	75.9	76.2	75.9	76.2	72.1	74.7	93.8	99.8	**	96.3
L.major [USA] AF109875	76.2	76.4	76.2	76.4	75.0	73.6	92.9	96.3	96.3	**

Table 4.2: Oligopeptidase B gene for different species as submitted by various workers

Sl. No.	Identity of *opdB* gene for *Trypanosoma evansi*	Accession No.	Collection Country	Reference
1.	*T. evansi*	JQ909240	India	Kumar *et al.* (2012b)
2.	*T. brucei*	AF078916	USA	Morty *et al.* (1999c)
3.	*T. evansi*	AY546084	Germany	Morty *et al.* (2005b)
4.	*T. brucei*	XM824253	UK	Berriman *et al.* (2008)
5.	*T. cruzi*	XM804874	USA	El-Sayed *et al.* (2008c)
6.	*T. cruzi*	U69897	USA	Burleigh *et al.* (1998)
7.	*L. amazonensis*	EF392367	Brazil	Matos Guedes *et al.* (2007b)

8.	*L. donovani*	GQ491028	USA	Swenerton *et al.* (2011)
9.	*L. infantum*	XM001463502	UK	Peacock *et al.* (2011a)
10.	*L. major*	AF109875	USA	Morty *et al.* (1999d)

```
T.cruzi_XM804874           ---------MKCGPIATPKDHEVVFGYVEGENRGNNAMNPPRRRNDPLFW 41
T.cruzi_U69897             ---------MKCGPIATPKDHEVVFGYVEGENRGNNAMNPPRRRNDPLFW 41
T.evansi_JQ909240          -------MQTERGPIAAHRPHEVVFGKVEGLDRGANPMDPPRRRVDPLFW 43
T.evansi_AY546084          -------MQTERGPIAAHRPHEVVFGKVEGLDRGANPMDPPRRRVDPLFW 43
T.brucei_AF078916          -------MQTERGPIAAHRPHEVVFGKVEGEDRGANPMDPPRRRVDPLFW 43
T.brucei_XM824253          -------MQTERGPIAAHRPHEVVFGKVEGEDRGANPMDPPRRKVDPLFW 43
L.donovani_GQ491028        MLSGNTIAAPAQPPIAAKKPHRVTFGYVEGEDRGPNPMNPPRYREDPYFW 50
L.infantum_XM001463502     MLSGNTIAASAQPPIAAKKPHRVTFGYVEGEDRGPNPMNPPRYREDPYFW 50
L.major_AF109875           MSSDSSVAASAQPPIAAKKPHRVKFGYVEGEDRGPNPMNPPRYREDPYFW 50
L.amazonensis_EF392367     MSSDSSVAASVQPPIAAKKPHRVTFGYVEGEDRGPNPMNPPRHHEDPYFW 50
                                     ***: : *.* ** *** :** *.*:*** : ** **

T.cruzi_XM804874           LRDDSRKNPEVIAHLKLEQAYFEERTADIKDFSETIFKEYISHIKETDIS 91
T.cruzi_U69897             LRDDSRKNPEVIAHLKLEQAYFEERTADIKDFSETIFKEYFSHIKETDIS 91
T.evansi_JQ909240          LRDDNRADPEVLAHLHLEKDYYEKRAVDIKDLAETIYQEHISHIEETDMS 93
T.evansi_AY546084          LRDDNRADPEVLAHLHLEKDYYEKRAVDIKDLAETIYQEHISHIEETDMS 93
T.brucei_AF078916          LRDDNRADPEVLAHLHLEKDYYEKRAVDIKDLAETIYQEHISHIEETDMS 93
```

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T.brucei_XM824253          LRDDNRADPEVLAHLHLEKDYYEKRAVDIKDLAETIYQEHISHIEETDMS 93
L.donovani_GQ491028        MRDDNRKDPAVIEHLNKEKAYFQARSADIAQLRDDIYTEHISHIKEDDMS 100
L.infantum_XM001463502     MRDDNRKDPAVIEHLNKEKAYFQARSADIAQLRDDIYTEHISHIKEDDMS 100
L.major_AF109875           MRDDDRKDPAVIEHLNKEKVYFQARSADIAQLRDDIYAEHISHINEDDMS 100
L.amazonensis_EF392367     MRDDDRKDPAVIEHLKKEKAYFEACSSDMTQLRDDIYTEHISHIKEDDMS 100
                           :***.* :* *: **: *: *::  : *: :: : *: *::***:* *:*

T.cruzi_XM804874           APYRYDGYTYYTREVEGLSYKIHCRVPLGMTPGKSEDEQVILDENKLAEG 141
T.cruzi_U69897             APYRYDGYTYYTREVEGLSYKIHCRVPLGMTPGKSEDEQVILDENKLAEG 141
T.evansi_JQ909240          APYVYDRFLYYTRDVKGLSYKLHCRVPAGKTPGEGEDEEIVLDENKLAEG 143
T.evansi_AY546084          APYVYDRFLYYTRDVKGLSYKLHCRVPAGKTPGEGEDEEIVLDENKLAEG 143
T.brucei_AF078916          APYVYDRFLYYTRDVKGLSYKLHCRVPAGKTPGEGEDEEIVLDENKLAEG 143
T.brucei_XM824253          APYVYDRFLYYTRDVKGLSYKLHCRVPAGKTPGEGEDEEIVLDENKLAEG 143
L.donovani_GQ491028        APYVYGKYRYYTREVKGKSYKIYCRVSKDKEPGDVAAEEVIIDVNQVVEG 150
L.infantum_XM001463502     APYVYGKYRYYTREVKGKSYKIYCRVSKDKEPGDVAAEEVIIDVNQVAEG 150
L.major_AF109875           APYVYGKYRYYTREVKGKPYKIYCRVFTDKEPGDVAAEEVIIDVNQVAEG 150
L.amazonensis_EF392367     APYLYGQYRYYTREVKGKSYKIYCRVPKDKEPGDVAVEEVVIDVNKVAEG 150
                           *** *. : ****:*:* .**::***  .  **.   *::::* *::.**

T.cruzi_XM804874           KPFCMVHEVKPAPPDHNLVAYSVDYIGNEMYTIRFTDN-----AVTDVVE 186
T.cruzi_U69897             KSFCMVHEVKPAPPDHNLVAYSVDYVGNEMYTIRFTDN-----AVTDVVE 186
T.evansi_JQ909240          KSFCVVGCVAPAPPEHALVAYSVDYCGDEVYSIRFVRD-----VVADKVE 188
T.evansi_AY546084          KSFCVVGCVAPAPPEHALVAYSVDYCGDEVYSIRFVRD-----VVADKVE 188
T.brucei_AF078916          KSFCVVGCVAPAPPEHALVAYSVDYCGDEVYSIRFVRD-----VVADKVE 188
T.brucei_XM824253          KSFCVVGCVAPAPPEHALVAYSVDYCGDEVYSIRFVRD-----VVADKVE 188
L.donovani_GQ491028        KPFCDVMEVKPAPPEHDLVAFSVDMSGNEVYTIEFKRISDPCQTIADKVS 200
L.infantum_XM001463502     KPFCDVMEVKPAPPEHDLVAFSVDMSGNEVYTIEFKRISDPCQTIADKVS 200
L.major_AF109875           KAFCDVMEVKPAPPEHDLVAFSVDMSGNEVYTIEFKRISDPSQTIADKVS 200
L.amazonensis_EF392367     KPFCDVMEVEPAPPEHDLVAFSVDMSGNEVYAIEFKHMSDPCRTVADTVS 200
                           *.** *  * ****:* ***:***  *:*:*:*.*        .::* *.

T.cruzi_XM804874           GTNGQILWGPNASCFFYMTKDAAERDYKIWRHIIGRPQSEDVCLYTENDL 236
T.cruzi_U69897             GTNGQILWGPNASCFFYITKDAAERNYKIWRHIIGRPQSEDVCLYTENDL 236
T.evansi_JQ909240          GTNGSVVWGPNAECFFYITKDASKRDNKVWRHIIGQPQSEDVCLYTDDDP 238
T.evansi_AY546084          GTNGSVVWGPNAECFFYITKDASKRDNKVWRHIIGQPQSEDVCLYTDDDP 238
T.brucei_AF078916          GTNGSVVWGPNAECFFYITKDASKRDNKVWRHIIGQPQSEDVCLYTDDDP 238
T.brucei_XM824253          GTNGSVVWGPNAECFFYITKDASKRDNKVWRHIIGQPQSEDVCLYTDDDP 238
L.donovani_GQ491028        GTNGEIVWGPDQTSLFYVTKDETLRDNKVWRHVMGKPQSEDVCLYEENNP 250
L.infantum_XM001463502     GTNGEIVWGPDQTSLFYVTKDETLRDNKVWRHVMGKPQSEDVCLYEENNP 250
L.major_AF109875           GTNGEIVWGPDHTSLFYVTKDETLRENKVWRHVMGKLQSEDVCLYEEHNP 250
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L.amazonensis_EF392367      GTNGEIVWGPDHTSFFYVTKDETLRENKVWRHVMGRPQSEDVCLYEENNP 250
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T.cruzi_XM804874            LFSTAMAISGDGSTLIIGSGSFETTESQLLDLRKGNGHTTLEMVRPREKG 286
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T.evansi_JQ909240           LFSVGVGRSGDGKTLIICSMSSETSESHLLDLRKGVKHNTLEMVRPREKG 288
T.evansi_AY546084           LFSVGVGRSGDGKTLIICSMSSETSESHLLDLRKGVKHNTLEMVRPREKG 288
T.brucei_AF078916           LFSVGVGRSGDGKTLIICSMSSETSESHLLDLRKGVKHNTLEMVRPREKG 288
T.brucei_XM824253           LFSVGVGKSGDGKTLIICSMSSETSESLLLDLRKGVKHNTLEMVRPREKG 288
L.donovani_GQ491028         LFSAFMYKAADTNTLCIGSQSPETAEVHLLDLRRGNAHNTLEIVRPREKG 300
L.infantum_XM001463502      LFSAFMYKAADTNTLCIGSQSPETAEVHLLDLRRGNAHNTLEIVRPREKG 300
L.major_AF109875            LFSAFMYKAADTNTLCIGSQSPETAEVHLLDLRKGNAHNTLEIVRPREKG 300
L.amazonensis_EF392367      LFSAFMYKAADTNTLCIGSQSSETTEVHLLDLRQGNAHNTVEVVRPREKG 300
                            ***. :  :.* .** * * * **:*  *****:*  *.*:*:*****:*

T.cruzi_XM804874            VRYDVELHGTENLLILTNKDNCMNGKVVLATRNTPSDWENIIVPHSEDVF 336
T.cruzi_U69897              VRYDVELHGTETLLILTNKDNCMNGKVVSATRNTPSDWENVIVPHSEDVF 336
T.evansi_JQ909240           VRYTVEMHGTDTLIVLTNKDKCVNGKVVLTKRSAPTDWGTVLIPHDDKVT 338
T.evansi_AY546084           VRYTVEMHGTDTLIVLTNKDKCVNGKVVLTKRSAPTDWGTVLIPHDDKVT 338
T.brucei_AF078916           VRYTVEMHGTDTLIVLTNKDKCVNGKVVLTKRSAPTDWGTVLIPHDDKVT 338
T.brucei_XM824253           VRYTVEMHGTDTLIVLTNKDKCVNGKVVLTKRSAPTDWGTVLIPHDDKVT 338
L.donovani_GQ491028         VRYDVQMHGTSHLVILTNEGGAVNHKLLIAPRGQPSDWSHVLVDHSEDVF 350
L.infantum_XM001463502      VRYDVQMHGTSHLVILTNEGGAVNHKLLIAPRGQPSDWSHVLVDHSEDVF 350
L.major_AF109875            VRYDVQMHGTSHLVILTNEGGAVNHKLLIAPRGQPSDWSHVLVDHSEDVF 350
L.amazonensis_EF392367      VRYNVQMHGTRHLLILTNKDGAVNHKLLIAPRGQPSDWSHVLVDHTEDVF 350
                            *** *::***  *::***:. .:* *:: : *. *:**  ::: * :.*

T.cruzi_XM804874            IGEIGVFAKFAVLSGRRGGLTRVWTMPVGS-DGLFRSGAFVQEVSFDEPV 385
T.cruzi_U69897              IGEIGVFAKFVVLSGRRGGITRVWAMPVGS-DGLFRSGAFVQEVSFDEPV 385
T.evansi_JQ909240           IDDVVVFAKFAVLSGRRDGLTRVWTVRLGP-DNLFSS-ATLKELHFDEPV 386
T.evansi_AY546084           IDDVVVFAKFAVLSGRRDGLTRVWTVRLGP-DNLFSS-ATLKELHFDEPV 386
T.brucei_AF078916           IDDVAVFAKFAVLSGRRDGLTRVWTVRLGP-DNLFSS-ATLKELHFDEPV 386
T.brucei_XM824253           IDDVAVFAKFAVLSGRRDGLTRVWTVRLGP-DSLFSS-ATLKELHFDEPV 386
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L.infantum_XM001463502      MESIAVRSNYLVVTGRRAGLTRIWTMMVDPQDGVFKASTGLREVVMEEPI 400
L.major_AF109875            MESIAVRSNYLVVAGRRAGLTRIWTMMADSQDGVFQAGTGLREVVMEEPI 400
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L.amazonensis_EF392367     MENIAVRSNYLVVTGRRGGLTRIWTMMVDPQDGVFKPGAELREVMMEEPI 400
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T.evansi_JQ909240          FTAHVVCSQMKTYDASLLRLRYSSMTTPTVWYDEDVLSGERKVVKARKVG 436
T.evansi_AY546084          FTAHVVCSQMKTYDASLLRLRYSSMTTPTVWYDEDVLSGERKVVKARKVG 436
T.brucei_AF078916          FTAHVVCSQMKTYDASLLRLRYSSMTTPTVWYDEDVLSGERKVVKARKVG 436
T.brucei_XM824253          FTAHVVCSQMKTYDASLLRLRYSSMTTPTVWYDEDVLSGERKVVKARKVG 436
L.donovani_GQ491028        FTVHLVAFQMLEYEEPTFRMEYSSLATPNTWLDVNPQDHSRTAVKVREVG 450
L.infantum_XM001463502     FTVHLVAFQMLEYEEPTFRMEYSSLATPNTWLDVNPQDHSRTAVKVREVG 450
L.major_AF109875           FTVHLVESQMLEYEEPTFRMEYSSLATPNTWFDVSPQDTLAPLVKVREVG 450
L.amazonensis_EF392367     FTVHLVESQMLEYEESTFRMEYSSLATPNTWFNVSPQDHSRTVVKVREVG 450
                           **.. *  :*  *:   :*: *:*::**..* :    .     **.*:*

T.cruzi_XM804874           GNFDAKNYTCRRLFATAPDRTKIPLSIVHDVSLDMSKPHPTVLYAYGSYG 485
T.cruzi_U69897             GNFDAKNYTCRRLFATAPDRTKIPLSIVHDVSLDMSKPHPTVLYAYGSYG 485
T.evansi_JQ909240          GGFESKNYVCRRELATAPDGTKVPISLVYDTSIDLKKPNPTMLYGYGSYG 486
T.evansi_AY546084          GGFESKNYVCRRELATAPDGTKVPISLVYDTSIDLKKPNPTMLYGYGSYG 486
T.brucei_AF078916          GGFESKNYVCRRELATAPDGTKVPISLVYDTSIDLKKPNPTMLYGYGSYG 486
T.brucei_XM824253          GGFQSKNYVCRRELATAPDGTKVPISLVYDTSIDLKKPNPTMLYGYGSYG 486
L.donovani_GQ491028        GGFDAANYKVERRFATAPDQTKIPLSVVYHKDLDMSQPQPCMLYGYGSYG 500
L.infantum_XM001463502     GGFDAANYKVERRFATAPDQTKIPLSVVYHKDLDMSQPQPCMLYGYGSYG 500
L.major_AF109875           GGFDAANYKVERRFATAPDQTKIPLSVVCHKDLDMSQPQPCMLYGYGSYG 500
L.amazonensis_EF392367     GGFDAANYKVERRFATAPDQTKIPLSLVYHKDLDMTQPQPCMLYGYGSYG 500
                           *.*:: **  .* :***** **:*:*:* . .:*:.:*:* :**.*****

T.cruzi_XM804874           VCVEPEFSVKYLPYLDRGVIYVIAHVRGGGEMGRAWYEVGAKYLTKRNTF 535
T.cruzi_U69897             ACVEPEFSVKYLPYLDRGVIYVIAHVRGGGEMGRAWYEVGAKYLTKRNTF 535
T.evansi_JQ909240          ICIEPEFNSRFLPYVDRGMIYAIAHVRGGGEMGRTWYEVGGKYLTKRNTF 536
T.evansi_AY546084          ICIEPEFNSRFLPYVDRGMIYAIAHVRGGGEMGRTWYEVGGKYLTKRNTF 536
T.brucei_AF078916          ICIEPEFNSRFLPYVDRGMIYAIAHVRGGGEMGRTWYEVGGKYLTKRNTF 536
T.brucei_XM824253          ICIEPEFNSRFLPYVDRGMIYAIAHVRGGGEMGRTWYEVGGKYLTKRNTF 536
L.donovani_GQ491028        LSMDPQFSIQHLPYCDRGMIFAIAHIRGGSEMGRAWYEIGAKYLTKRNTF 550
L.infantum_XM001463502     LSMDPQFSIQHLPYCDRGMIFAIAHIRGGSEMGRAWYEIGAKYLTKRNTF 550
L.major_AF109875           LSMDPQFSIQHLPYCDRGMIFAIAHIRGGSELGRAWYEIGAKYLTKRNTF 550
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L.amazonensis_EF392367     ISMDPQFTIQHLPYCDRGMIYVIAHIRGGSEMGRAWYEIGAKYLTKRNTF 550
                            .::*:*. :.*** ***:*:.***:***.*:**:***:*.********

T.cruzi_XM804874           SDFIACAEYLIEIGLTTPSQLACEGRSAGGLLIGAVLNMRPDLFRVALAG 585
T.cruzi_U69897             SDFIACAEYLIEIGLTTPSQLACEGRSAGGLLIGAVLNMRPDLFRVALAG 585
T.evansi_JQ909240          MDFIACAEHLISSGLTTPAQLSCEGRSAGGLLVGAVLNMRPDLFHVALAG 586
T.evansi_AY546084          MDFIACAEHLISSGLTTPAQLSCEGRSAGGLLVGAVLNMRPDLFHVALAG 586
T.brucei_AF078916          MDFIACAEHLISSGLTTPAQLSCEGRSAGGLLVGAVLNMRPDLFHVALAG 586
T.brucei_XM824253          MDFIACAEHLISSGLTTPAQLSCEGRSAGGLLVGAVLNMRPDLFHVALAG 586
L.donovani_GQ491028        SDFIAAAEFLVNAKLTTPSQLACEGRSAGGLLVGAVLNMRPDLFKVALAG 600
L.infantum_XM001463502     SDFIAAAEFLVNAKLTTPSQLACEGRSAGGLLVGAVLNMRPDLFKVALAG 600
L.major_AF109875           SDFIAAAEFLVNAKLTTPSQLACEGRSAGGLLMGAVLNMRPDLFKVALAG 600
L.amazonensis_EF392367     SDFIAAAEFLVDAKLTTPSQLACEGRSAGGLLVGAVLNMRPDLFKVALAG 600
                            ****.**.*:.  ****:**:**********:***********:*****

T.cruzi_XM804874           VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPVDNVRAQD 635
T.cruzi_U69897             VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPVDNVRAQD 635
T.evansi_JQ909240          VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNARAQD 636
T.evansi_AY546084          VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNARAQD 636
T.brucei_AF078916          VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNVRAQD 636
T.brucei_XM824253          VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNVRAQD 636
L.donovani_GQ491028        VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKYYDYMLSYSPVDNVRAQE 650
L.infantum_XM001463502     VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKYYDYMLSYSPVDNVRAQE 650
L.major_AF109875           VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKYYDYMLSYSPMDNVRAQE 650
L.amazonensis_EF392367     VPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKYYDYMLSYSPMDNVRAQE 650
                           *********************************::*** ****:**.***:

T.cruzi_XM804874           YPHLMIQAGLHDPRVAYWEPAKWASKLRALKTDSNEVLLKMDLESGHFSA 685
T.cruzi_U69897             YPHLMIQAGLHDPRVAYWEPAKWASKLRALKTDSNEVLLKMDLESGHFSP 685
T.evansi_JQ909240          YPHLMIQAGLHDPRVAYWEPAKWASKLRELKTDSNEVLLKMDLESGHFSA 686
T.evansi_AY546084          YPHLMIQAGLHDPRVAYWEPAKWASKLRELKTDSNEVLLKMDLESGHFSA 686
T.brucei_AF078916          YPHLMIQAGLHDPRVAYWEPAKWASKLRELKTDSNEVLLKMDLESGHFSA 686
T.brucei_XM824253          YPHLMIQAGLHDPRVAYWEPAKWASKLRELKTDSNEVLLKMDLESGHFSA 686
L.donovani_GQ491028        YPNIMVQCGLHDPRVAYWEPAKWVSKLRECKTDNNEILLNMDMESGHFSA 700
L.infantum_XM001463502     YPNIMVQCGLHDPRVAYWEPAKWVSKLRECKTDNNEILLNMDMESGHFSA 700
L.major_AF109875           YPNIMVQCGLHDPRVAYWEPAKWVSKLRECKTDNNEILLNIDMESGHFSA 700
L.amazonensis_EF392367     YPNIMVQCGLHDPRVAYWEPAKWVSKLREHKTDCNEILLNMDMESGHFSA 700
                           **::*:*.***************.****  *** **:**::*:******.

T.cruzi_XM804874           SDRYRYWREMSFQQAFVLKHLN--ARTLLRR 714
```

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T.cruzi_U69897              SDRYRYWREMSFPQAFVLKHLN--ARTLLRR 714
T.evansi_JQ909240           SDRYKYLRENA-------------------- 697
T.evansi_AY546084           SDRYKYLRENAIQQAFVLKHLN--VRQLLRK 715
T.brucei_AF078916           SDRYKYLRENAIQQAFVLKHLN--VRQLLRK 715
T.brucei_XM824253           SDRYKYLRENAIQQAFVLKHLN--VRQLLRK 715
L.donovani_GQ491028         KDRYKFWKESAIQQAFVCKHLKSTVRLLVRR 731
L.infantum_XM001463502      KDRYKFWKESAIQQAFVCKHLKSTVRLLVRR 731
L.major_AF109875            KDRYKFWKESAIQQAFVCKHLKSTVRLLVRR 731
L.amazonensis_EF392367      RDRYKFWKESAVQQAFVCKHLKSTVRLLVRR 731
                              ***:: :* :
```

Plate 23. Multiple sequence alignment of Oligopeptidase B Amino acid sequences with Clustal W.

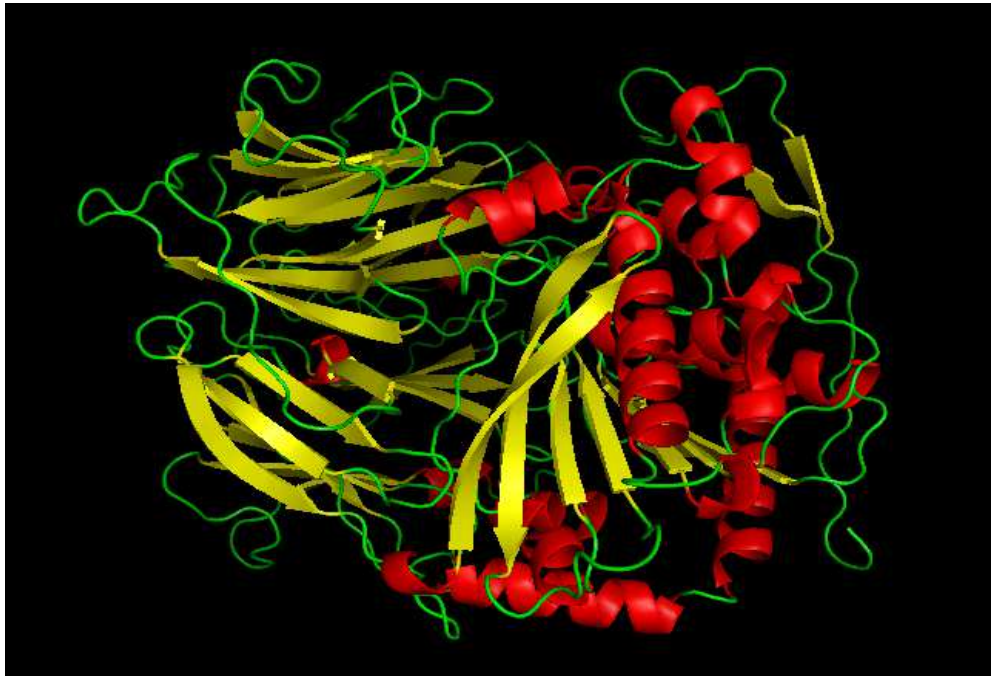

Plate 24a. A putative 3D model structure of Oligopeptidase B protein with ribbon representation

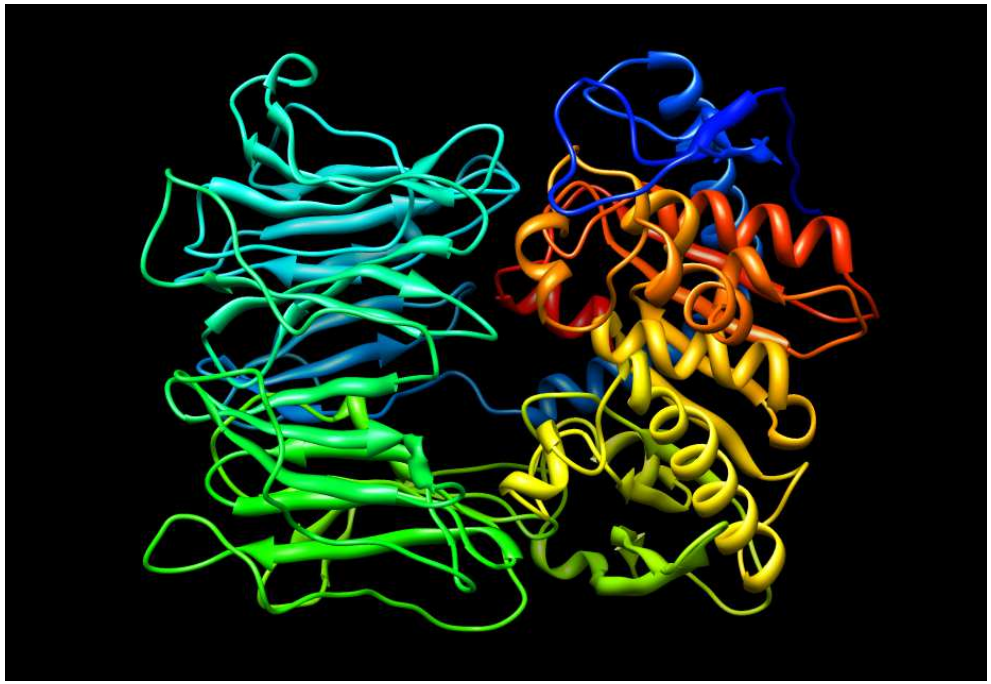

Plate 24b. Cartoon representation of 3D model of Oligopeptidase B protein

ORIGIN

```
  1 ATGGCCGCAG TTGACGATGC CACTGGTTTG GAGGCTGCGC GCAAGCAGAA GATCCACAAC
 61 CTGAAGCTGA AGACAGCCTG TTTGGAGAAT GAGGAACTTG TACAGGAATT GCATGTATCT
121 GACTGGAGCG AGACACAGAG GCAGAAGCTG CGCGGCGCCC ACCTGAAGGC TGAGGAGCTG
181 GTTGCCGCTG TGGACGTCGG TACGAAATGG AACCTAACGG AGGTATACGA CCTCGCAAAG
241 CTGATGCGCG TGTGTGGACT CGAGATGAGC CAACGCGAGC TTTACCGCCC TGAGGACAAG
301 GCACAATTCA TGGACATTAT TGCCATGAAA AAGGTGCTTC AGGACCTGCG TCAGAACCGC
```

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 361 AACAAGACGC GTGTTGTGAG CTTCACGCAG ATGATCGACA ACGCCATCGC GAAGGTTGAA
 421 AAGGTTGAGG AGGAGCTTCG CCGCTCGCAG CTGGATGCAA CACAGTTGGC GCAGGTCCCC
 481 ACACAGACAT TGAAGCAAGT GGAGGATATC ATGAACGTAA CGCAAATCCA GAATGCGCTT
 541 GCCTCAACTG ACGACCAGAT CAAGACGCAG TTGGCGCAGC TTGAAAAAAC GAACGAGATC
 601 CAGAACGTTG CGATGCATGA TGGTGAGATG CAGGTCGCCG AGGAGCAAAT GTGGACGAAG
 661 GTACAGCTTC AGGAGCGCTT GATCGATCTG ATTCAGGACA AATTCCGCTT GATCAGCAAA
 721 TGTGAGGAGG AGAACCAGGC CTTCAGCAAA ATCCATGAGG TGCAGAAACA GGCGAATCAG
 781 GAAACGAGTC AGATGAAGGA TGCGAAGCGT CGCCTGAAGC AGCGGTGTGA GACAGATCTG
 841 AAGCACATCC ACGACGCGAT CCAGAAGGCT GACCTTGAGG ATGCCGAGGC GACGAAGCGC
 901 CACGCTGCGA ACAAAGAGAA GAGCGACCGC TACATCCGAG AGAACGAGGA TAGGCAGGAG
 961 GAGACGTGGA ACAAGATCCA GGACCTTGAG CGGCAGTTGC AGAAGCTTGG CACGGAGCGA
1021 TTCGATGAGG TCAAGCGGCG GATTGAGGAG ATTGACCGCG AGGAGAAGCG ACGTGTGGAG
1081 TACTCTCAAT TCCTGGAGGT TGCCTCGCAG CACAAGAAAC TGCTCGAGCT GACAGTGTAC
1141 AACTGCGACC TCGCGATCCG CTGTACCGGG CTGGTGGAGG AGCTGGTGTC GGAGGGCTGT
1201 GCCGCGGTGA AGGCCCGCCA CGACAAAACG AGCCAGGATC TTGCAGCCCT TCGTTTGGAT
1261 GTTCATAAAG AGCACTTGGA GTACTTCCGC ATGCTGTACC TCACGTTGGG TTCTCTTATC
1321 TACAAGAAAG AGAAGCGGAT GGAGGAGATT GACCGGAACA TCCGTACAAC GCACATCCAG
1381 TTGGAGTTCT GTGTGGAAAC ATTCGACCCG AATGCGAAGA AGCACGCCGA CATGAAGAAA
1441 GAGCTATACA GGCTGCGCCA GGGCGTAGAG GAGGAGCTGG CGATGCTGAA AGAGAAGCAG
1501 GCGAAGGCGT TGGAGGAGTT CAAGGAGTCA GAGGAGGCTC TGGACGCTGC TGGCATCGAG
1561 TTCAACCACC CTGTGGACGA GAACAACGAG GAGGTGCTTA CACGCCGCAG CAAGATGGTG
1621 GAGTACCGCT CGCACCTGAC GAAGCAGGAG GAGGTGAAGA TTGCCGCCGA ACGCGAGGAA
1681 ATCAAGAGGG CGCGCTTACT GCGCAGCTCT GGTGCCGGTG GCGAGCAGGT CCGCATCGGA
1741 AACAACACTG CACCGGCACG CCTCGAATA
```

Plate 25. The coding sequence of Paraflagellar rod 1 gene of *T. evansi* isolated from *Camelus dromedarius*

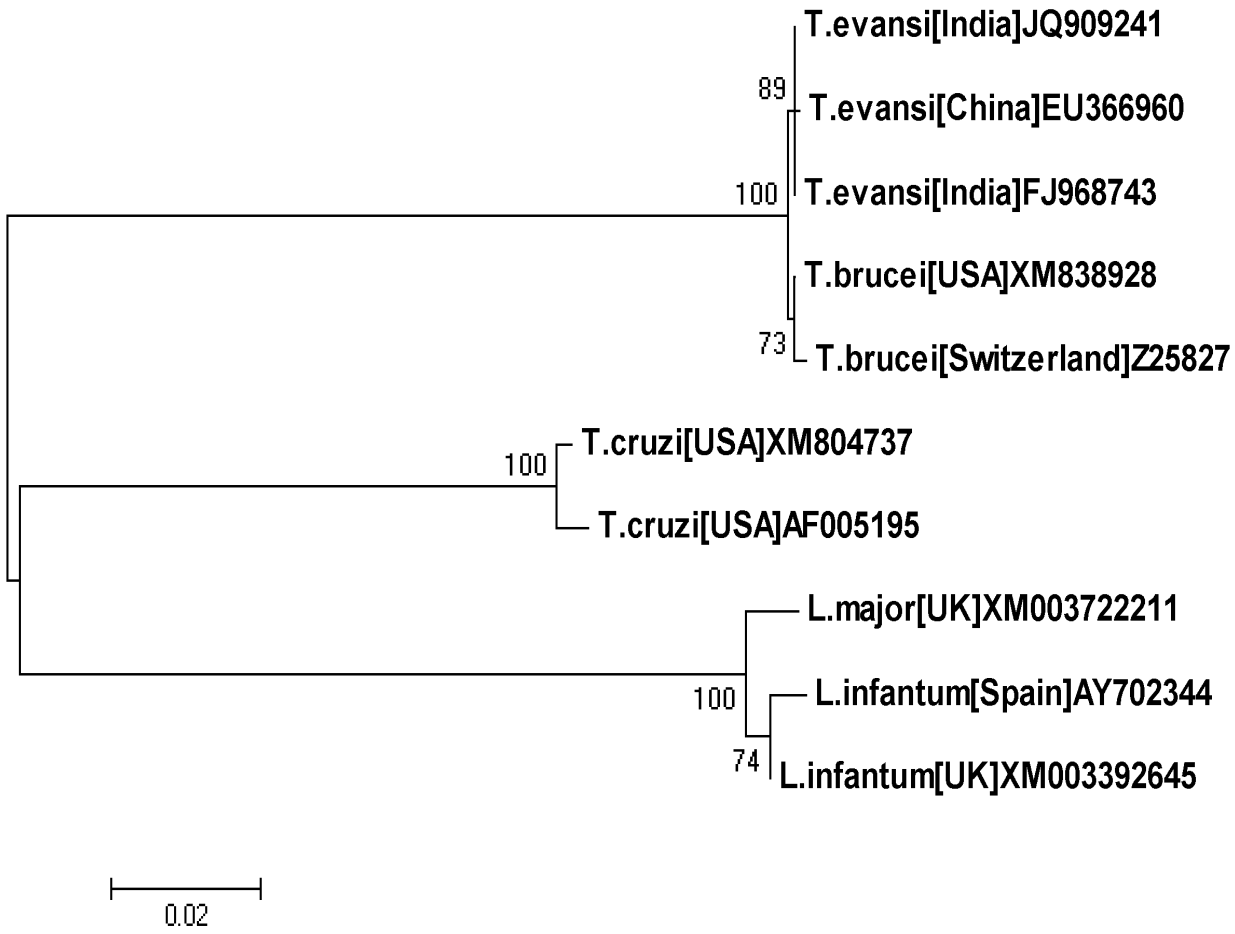

Plate 26a. Phylogenetic tree analysis of Paraflagellar rod 1 gene using the Neighbor-Joining method

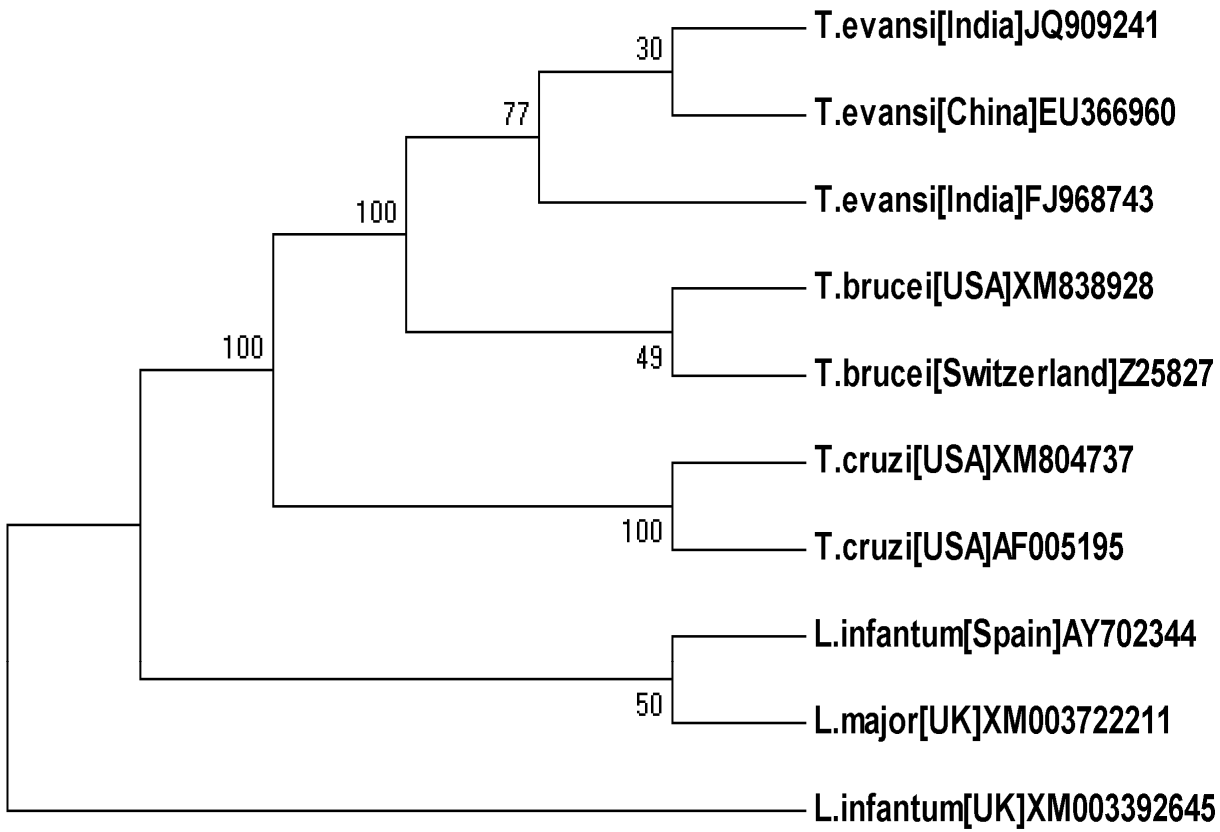

Plate 26b. Phylogenetic tree analysis of Paraflagellar rod 1 gene using maximum parsimony method

Table 4.3: Sequence identity of Paraflagellar rod 1 gene of *T. evansi* with other allied species

Identity of *pfr*1 gene for *Trypanosoma evansi*	*T.evansi* [India] JQ909241	*T.evansi* [China] EU366960	*T.evansi* [India] FJ968743	*T.brucei* [USA] XM838928	*T.brucei* [Switzerland] Z25827	*T.cruzi* [USA] XM804737	*T.cruzi* [USA] AF005195	*L.infantum* [Spain] AY702344	*L.major* [UK] XM003722211	*L.infantum* [UK] XM003392645
T.evansi [India]	**	99.9	100	99.8	98.4	84.4	84.2	83.6	83.6	83.9

JQ909241										
T.evansi [China] EU366960	99.9	**	99.9	99.8	98.4	84.3	84.1	83.5	83.6	83.8
T.evansi [India] FJ968743	100	99.9	**	99.8	99.4	84.4	84.2	83.6	83.6	83.9
T.brucei [USA] XM838928	99.8	99.8	99.8	**	99.5	84.3	84.1	83.6	83.6	83.9
T.brucei [Switzerland] Z25827	99.4	99.4	99.4	99.5	**	84.8	84.7	83.3	83.4	83.6
T.cruzi [USA] XM804737	84.4	84.3	84.4	84.3	84.8	**	99.4	86.2	86.1	86.5
T.cruzi [USA] AF005195	84.2	84.1	84.2	84.1	84.7	99.4	**	86.0	85.9	86.3
L.infantum [Spain] AY702344	83.6	83.5	83.6	83.6	83.3	86.2	86.0	**	98.8	99.6
L.major [UK] XM003722211	83.6	83.6	83.6	83.6	83.4	86.1	85.9	98.8	**	98.8
L.infantum [UK] XM003392645	83.9	83.8	83.9	83.9	83.6	86.5	86.3	99.6	98.8	**

Table 4.4: Paraflagellar rod 1 gene for different species as submitted by various workers

Sl. No.	Identity of *pfr1* gene for *Trypanosoma evansi*	Accession No.	Collection Country	Reference
1.	*T. evansi*	JQ909241	India	Kumar *et al.* (2012c)
2.	*T.evansi*	EU366960	China	Abdille *et al.* (2008b)
3.	*T.evansi*	FJ968743	India	Maharana *et al.* (2009a)
4.	*T.brucei*	XM838928	USA	Berriman *et al.* (2012)
5.	*T.brucei*	Z25827	Switzerland	Deflorin *et al.* (2004)

6.	*T.cruzi*	XM804737	USA	El-Sayed *et al.* (2008a)
7.	*T.cruzi*	AF005195	USA	Fouts *et al.* (1998)
8.	*L.infantum*	AY702344	Spain	Waeger *et al.* (2004)
9.	*L.major*	XM003722211	UK	Rogers *et al.* (2012)
10.	*L.infantum*	XM003392645	UK	Peacock *et al.* (2011b)

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T.cruzi_XM804737          -MSAEEATGLEAARKQKIHNLKLKTACLENEELIQELHVSDWSETQRQKL 49
T.cruzi_AF005195          -MSAEEATGLEAARKQKIHNLKLKTACLENEELIQELHVSDWSETQRQKL 49
T.evansi_JQ909241         MAAVDDATGLEAARKQKIHNLKLKTACLENEELVQELHVSDWSETQRQKL 50
T.evansi_EU366960         MAAVDDATGLEAARKQKIHNLKLKTACLENEELVQELHVSDWSETQRQKL 50
T.evansi_FJ968743         MAAVDDATGLEAARKQKIHNLKLKTACLENEELVQELHVSDWSETQRQKL 50
T.brucei_XM838928         MAAVDDATGLEAARKQKIHNLKLKTACLENEELVQELHVSDWSETQRQKL 50
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T.brucei_Z25827            MAAVDDATGLEAARKQKIHNLKLKTACLENEELVQELHVSDWSETQRQKL 50
L.infantum_AY702344        MMTPEDATGLEAARKQKIHNLKLKTACLENEELVQELHISDWSETQRQKL 50
L.major_XM003722211        MMTPEDATGLEAARKQKIHNLKLKTACLENEELVQELHISDWSETQRQKL 50
L.infantum_XM003392645     MMAPEDATGLEAARKQKIHNLKLKTACLENEELVQELHISDWSETQRQKL 50
                             : ::****************************:****:***********

T.cruzi_XM804737           RGAHLKAEELVASVDVGTKWNLTEAYDLAKLMRVCGLEMSQRELYRPEDK 99
T.cruzi_AF005195           RGAHLKAEELVASVDVGTKWNLTEAYDLAKLMRVCGLEMSQRELYRPEDK 99
T.evansi_JQ909241          RGAHLKAEELVAAVDVGTKWNLTEVYDLAKLMRVCGLEMSQRELYRPEDK 100
T.evansi_EU366960          RGAHLKAEELVAAVDVGTKWNLTEVYDLAKLMRVCGLEMSQRELYRPEDK 100
T.evansi_FJ968743          RGAHLKAEELVAAVDVGTKWNLTEVYDLAKLMRVCGLEMSQRELYRPEDK 100
T.brucei_XM838928          RGAHLKAEELVAAVDVGTKWNLTEVYDLAKLMRVCGLEMSQRELYRPEDK 100
T.brucei_Z25827            RGAHLKAEELVAAVDVGTKWNLTEVYDLAKLMRVCGLEMSQRELYRPEDK 100
L.infantum_AY702344        RGAHEKGEELLASVEVGTKWNLMEAYDLAKLMRVCGLEMSQRELYRPEDK 100
L.major_XM003722211        RGAHEKGEELLASVEVGTKWNLMEAYDLAKLMRVCGLEMSQRELYRPEDK 100
L.infantum_XM003392645     RGAHEKGEELLASVEVGTKWNLMEAYDLAKLMRVCGLEMSQRELYRPEDK 100
                           **** *.***:*:*:******* *.************************

T.cruzi_XM804737           AQFMDIIGVKKVLQDLKQNRNKTRVVSFTQMIDNAIAKMEKVEEELRRSQ 149
T.cruzi_AF005195           AQFMDIIGVKKVLQDLKQNRNKTRVVSFTQMIDNAIAKMEKVEEELRRSQ 149
T.evansi_JQ909241          AQFMDIIAMKKVLQDLRQNRNKTRVVSFTQMIDNAIAKVEKVEEELRRSQ 150
T.evansi_EU366960          AQFMDIIAMKKVLQDLRQNRNKTRVVSFTQMIDNAIAKVEKVEEELRRSQ 150
T.evansi_FJ968743          AQFMDIIAMKKVLQDLRQNRNKTRVVSFTQMIDNAIAKVEKVEEELRRSQ 150
T.brucei_XM838928          AQFMDIIAMKKVLQDLRQNRNKTRVVSFTQMIDNAIAKVEKVEEELRRSQ 150
T.brucei_Z25827            AQFMDIIAMKKVLQDLRQNRNKTRVVSFTQMIDNAIAKVEKVEEDVRRSQ 150
L.infantum_AY702344        PQFMDIIGVKKVLQDLRQNRNKTRVVSFTQLIDNSIAKMEKVEEELRRSQ 150
L.major_XM003722211        PQFMDIIGVKKVLQDLRQNRNKTRVVSFTQLIDNSIAKMEKVEEELRRSQ 150
L.infantum_XM003392645     PQFMDIIGVKKVLQDLRQNRNKTRVVSFTQLIDNSIAKMEKVEEELRRSQ 150
                           .******.:*******:*************:***:***:*****::****

T.cruzi_XM804737           LDATQLAQVPTRTLKQIEDIMNATQIQNALASTDDQIKTQLAQLEKTNEI 199
T.cruzi_AF005195           LDATQLAQVPTRTLKQIEDIMNATQIQNALASTDDQIKTQLAQLEKTNEI 199
T.evansi_JQ909241          LDATQLAQVPTQTLKQVEDIMNVTQIQNALASTDDQIKTQLAQLEKTNEI 200
T.evansi_EU366960          LDATQLAQVPTQTLKQVEDIMNVTQIQNALASTDDQIKTQLAQLEKTNEI 200
T.evansi_FJ968743          LDATQLAQVPTQTLKQVEDIMNVTQIQNALASTDDQIKTQLAQLEKTNEI 200
T.brucei_XM838928          LDATQLAQVPTQTLKQVEDIMNVTQIQNALASTDDQIKTQLAQLEKTNEI 200
T.brucei_Z25827            LDATQLAQVPTQTLKQVEDIMNVTQIQNALASTDDQIKTQLAHVEKTNEI 200
L.infantum_AY702344        LDATQLAQVPTRTVKMMEDIMNATQIQNALASTDDQMQTQLAQLEKTNEI 200
L.major_XM003722211        LDATQLAQVPTRTVKMMEDIMNATQIQNALASTDDQMQTQLAQLEKTNEI 200
L.infantum_XM003392645     LDATQLAQVPTRTVKMMEDIMNATQIQNALASTDDQMQTQLAQLEKTNEI 200
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T.cruzi_XM804737          QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLITKCEEENQPFKK 249
T.cruzi_AF005195          QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLITKCEEENQPFKK 249
T.evansi_JQ909241         QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLISKCEEENQAFSK 250
T.evansi_EU366960         QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLISKCEEENQAFSK 250
T.evansi_FJ968743         QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLISKCEEENQAFSK 250
T.brucei_XM838928         QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLISKCEEENQAFSK 250
T.brucei_Z25827           QNVAMHDGEMQVAEEQMWTKVQLQERLIDLIQDKFRLISKCEEENQAFSK 250
L.infantum_AY702344       QNVAMHDGETQVAEEQMWTKVQLQERLIELLKDKFGLIGKCEEENAQFKE 250
L.major_XM003722211       QNVAMHDGEMQVAEEQMWTKVQLQERLIELLKDKFGLIGKCEEENAQFKE 250
L.infantum_XM003392645    QNVAMHDGEMQVAEEQMWTKVQLQERLIELLKDKFGLIGKCEEENAQFKE 250
                          ********* *****************:*::*** ** ******  *.:



T.cruzi_XM804737          IYEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEAMKR 299
T.cruzi_AF005195          IYEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEAMKR 299
T.evansi_JQ909241         IHEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEATKR 300
T.evansi_EU366960         IHEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQNADLEDAEATKR 300
T.evansi_FJ968743         IHEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEATKR 300
T.brucei_XM838928         IHEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEATKR 300
T.brucei_Z25827           IHEVQKQANQETSQMKDAKRRLKQRCETDLKHIHDAIQKADLEDAEATKR 300
L.infantum_AY702344       IYEVQKQANQETSQMKDAKRRLRQRCETDLKHIQDAIQKADLEDAEAVKR 300
L.major_XM003722211       IYEVQKQANQETSQMKDAKRRLRQRCETDLKHIQDAIQKADLEDAEAAKR 300
L.infantum_XM003392645    IYEVQKQANQETSQMKDAKRRLRQRCETDLKHIQDAIQKADLEDAEAVKR 300
                          *:********************:**********:****:******** **

T.cruzi_XM804737          HAANREKSDGFVRENEERQEEAWNKIQDLERQLQKLGTERFEEVKRRIEE 349
T.cruzi_AF005195          HAANREKSDGFVRENEERQEEAWNKIQDLERQLQKLGTERFEEVKRRIEE 349
T.evansi_JQ909241         HAANKEKSDRYIRENEDRQEETWNKIQDLERQLQKLGTERFDEVKRRIEE 350
T.evansi_EU366960         HAANKEKSDRYIRENEDRQEETWNKIQDLERQLQKLGTERFDEVKRRIEE 350
T.evansi_FJ968743         HAANKEKSDRYIRENEDRQEETWNKIQDLERQLQKLGTERFDEVKRRIEE 350
T.brucei_XM838928         HAANKEKSDRYIRENEDRQEETWNKIQDLERQLQKLGTERFDEVKRRIEE 350
T.brucei_Z25827           HAANKEKSDRYIRENEDRQEETWNKIQDLERQLQKLGTERFDEVKRRIEE 350
L.infantum_AY702344       YPRNKERSERAIKENEEMQEEAWNKIQDLERQLQNLGTDRFDEVKRRIEE 350
L.major_XM003722211       YAGNKERSERAIKENEEMQEEAWNKIQDLERQLQNLGTDRFDEVKRRIEE 350
L.infantum_XM003392645    YAGNKERSERAIKENEEMQEEAWNKIQDLERQLQNLGTDRFDEVKRRIEE 350
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T.cruzi_XM804737          VDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 399
T.cruzi_AF005195          VDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 399
T.evansi_JQ909241         IDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 400
T.evansi_EU366960         IDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 400
T.evansi_FJ968743         IDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 400
T.brucei_XM838928         IDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 400
T.brucei_Z25827           IDREEKRRVEYSQFLEVASQHKKLLELTVYNCDLAIRCTGLVEELVSEGC 400
L.infantum_AY702344       VDREEKRRVENAQFLEIAAQHKKLLELTVYNCDLAMRCTGLVEELVSEGC 400
L.major_XM003722211       VDREEKRRVENAQFLEIAAQHKKLLELTVYNCDLAMRCTGLVEELVSEGC 400
L.infantum_XM003392645    VDREEKRRVENAQFLEIAAQHKKLLELTVYNCDLAMRCTGLVEELVSEGC 400
                          :********* :****:*:****************:**************

T.cruzi_XM804737          AAVKARHDKTSQDLAALRLEVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 449
T.cruzi_AF005195          AAVKARHDKTSQDLAALRLEVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 449
T.evansi_JQ909241         AAVKARHDKTSQDLAALRLDVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 450
T.evansi_EU366960         AAVKARHDKTSQDLAALRLDVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 450
T.evansi_FJ968743         AAVKARHDKTSQDLAALRLDVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 450
T.brucei_XM838928         AAVKARHDKTSQDLAALRLDVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 450
T.brucei_Z25827           AAVKARHDKTSQDLAALRLDVHKEHLEYFRMLYLTLGSLIYKKEKRMEEI 450
L.infantum_AY702344       AGVKARYDKTNQDLAALRLEVHKEHLEYFRMLYLTLGSLIYKKEKRLEEI 450
L.major_XM003722211       AGVKARYDKTNQDLAALRLEVHKEHLEYFRMLYLTLGSLIYKKEKRLEEI 450
L.infantum_XM003392645    AGVKARYDKTNQDLAALRLEVHKEHLEYFRMLYLTLGSLIYKKEKRLEEI 450
                          *.****:***.********:***************************:***

T.cruzi_XM804737          DRNIRTTHIQLEFCVETFDPNAKRHADMKKELYKLRQGVEEELAMLKEKQ 499
T.cruzi_AF005195          DRNIRTTHIQLEFCVETFDPNAKRHADMKKELYKLRQGVEEELAMLKEKQ 499
T.evansi_JQ909241         DRNIRTTHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
T.evansi_EU366960         DRNIRTTHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
T.evansi_FJ968743         DRNIRTTHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
T.brucei_XM838928         DRNIRTTHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
T.brucei_Z25827           DRNIRTTHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
L.infantum_AY702344       DRNIRLAHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
L.major_XM003722211       DRNIRLAHIQLEFCVETFDPNAKKHADMKKELYKLRQGVEEELAMLKEKQ 500
L.infantum_XM003392645    DRNIRLAHIQLEFCVETFDPNAKKHADMKKELYRLRQGVEEELAMLKEKQ 500
                          ***** :****************:*********:****************
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T.cruzi_XM804737           AKALEDFKESEEALDAAGIEFNHPVDENNEEVLTRRSKMVEYRSHLSKQE 549
T.cruzi_AF005195           AKALEDFKESEEALDRAGIEFNHPVDENNEEVLTRRSKMVEYRSHLSKQE 549
T.evansi_JQ909241          AKALEEFKESEEALDAAGIEFNHPVDENNEEVLTRRSKMVEYRSHLTKQE 550
T.evansi_EU366960          AKALEEFKESEEALDAAGIEFNHPVDENNEEVLTRRSKMVEYRSHLTKQE 550
T.evansi_FJ968743          AKALEEFKESEEALDAAGIEFNHPVDENNEEVLTRRSKMVEYRSHLTKQE 550
T.brucei_XM838928          AKALEEFKESEEALDAAGIEFNHPVDENNEEVLTRRSKMVEYRSHLTKQE 550
T.brucei_Z25827            AKALEEFKESEEVSGRCWHRVQPPCDENNEEVLTRRSKMVEYRSHLTKQE 550
L.infantum_AY702344        AAALDDFKESEEALDAAGIEFSHPVDENNEEVLTRRSKMVEYKSHLTKEE 550
L.major_XM003722211        AAALDDFKESEEALDAAGIEFSHPVDENNEEVLTRRSKMVEYKSHLTKQE 550
L.infantum_XM003392645     AAALDDFKESEEALDAAGIEFSHPVDENNEEVLTRRSKMVEYKSHLTKQE 550
                           * **::******. . .  ... * ****************:***:*:*

T.cruzi_XM804737           EVKIAAEREEIKRAR------LLRTGGGGSGEQPRIGNN----TAPARLE- 589
T.cruzi_AF005195           EVKIAAEREEIKRAR------LLRTGGGGSGEQPRIGNN----TAPARLE- 589
T.evansi_JQ909241          EVKIAAEREEIKRAR------LLRSSGAG-GEQVRIGNN----TAPARLE- 589
T.evansi_EU366960          EVKIAAEREEIKRAR------LLRSSGAG-GEQVRIGNN----TAPARLE- 589
T.evansi_FJ968743          EVKIAAEREEIKRAR------LLRSSGAG-GEQVRIGNN----TAPARLE- 589
T.brucei_XM838928          EVKIAAEREEIKRAR------LLRSSGAG-GEQVRIGNN----TAPARLE- 589
T.brucei_Z25827            EVKIAAEREEIKRALTAQLWCRWRAGPHR-KQHCTHASN----SVAAIIM- 595
L.infantum_AY702344        EVRIAAEREEIKRAR------LLRSGGESAAAQITSGSMNADYAASAQLEL 595
L.major_XM003722211        EVRIAAEREEIKRAR------LLRSGGESAAAQITSGSMNADYAASAQLEL 595
L.infantum_XM003392645     EVRIAAEREEIKRAR------LLRSGGESAAAQITSGSMNADYAASA---- 591
                           **:***********         *:.      :   ..     :..*
```

Plate 27. Multiple sequence alignment of Paraflagellar rod 1 Amino acid sequences with Clustal W.

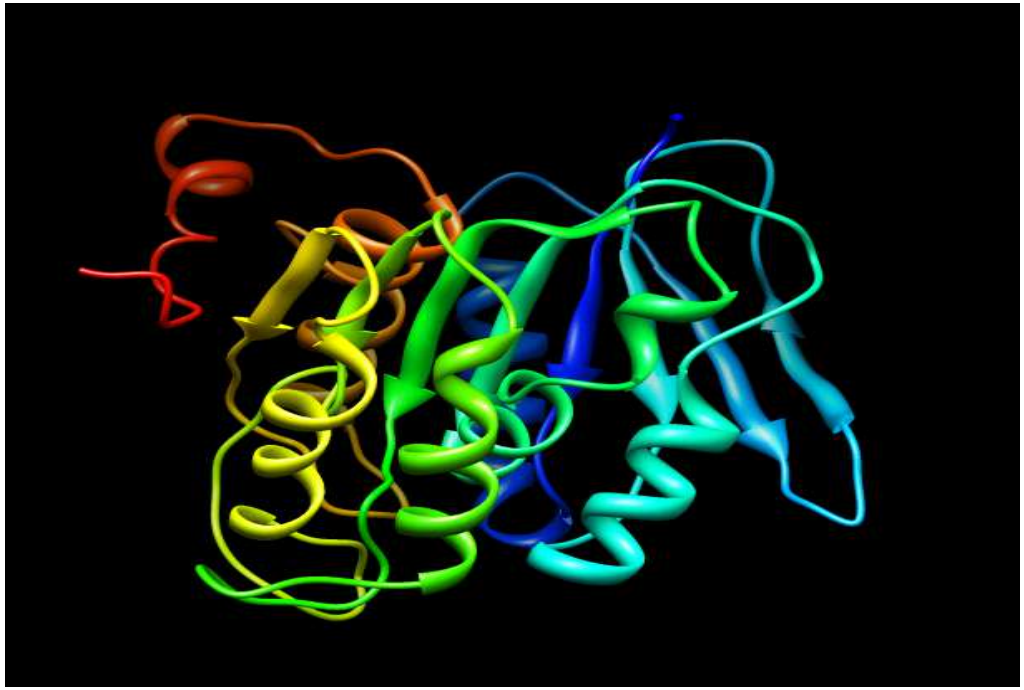

Plate 28a. Cartoon representation of 3D model of Paraflagellar rod 1 protein

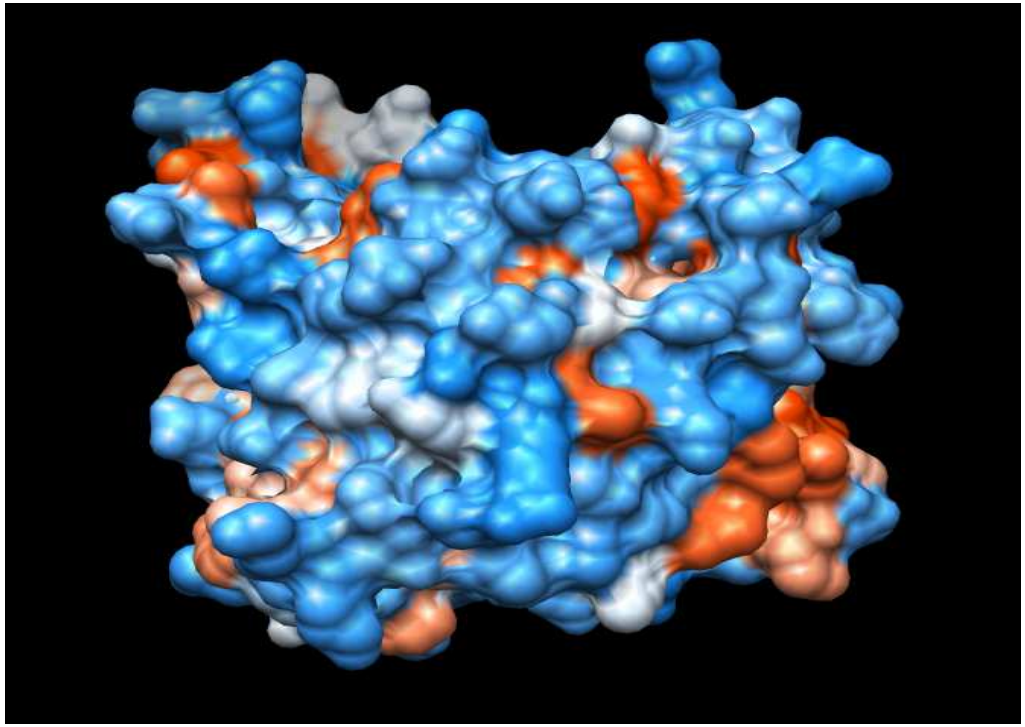

Plate 28b. Crystallographic 3D structure of Paraflagellar rod 1 protein

ORIGIN

```
  1 ATGAGCGGAA AGGAAGTTGA AGGTGTTGTG AGTCCTGCGG ACCAGCAGCA GCCAGCCGTC
 61 CCGGAGGTAA CAGATATCAC GCTGGAGGCC GCCCGCAAGC AGAAAATTCA CAACCTGAAG
121 TTGAAGACCG CCTGCCTTTC GAATGAGGAA TATGTCCAGG ACCTGCACGT ATCCGAGTGG
181 AGTGAGACGC AGAAGCAGAA GCTGCAGGCT GCACACGAGA AAGCGCATGA ATTGCTTGCC
241 TCAGTGGAGG GTGGGACGAA GTGGAGCCTG ACAGAGGCGT ATGACATCAA GAAGCTGATG
301 CGCGTCTGTG GTCTTGAGAT GTCTGTGCGT GAACTGTACA AGCCGGAGGA CAAGCCACAG
361 TTCATGGAGA TTGTTGCACT CAAGAAGACA ATGAACGAAC TGAAGCAACA TCACAACAAG
```

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 421 ACTCGCACGG TGTCTTTCAC CGGCATGATC GACAATGCCA TCGCCAAACT GGAGAAAATC
 481 GAAGACGAAC TGCGCCGGTC CCAGCTCGAC GCTTCTGAGA TGGCGCAAGT TCCTGTGGCT
 541 GCACTGAAGA ATATTGAGGA CACGATGAAC GTGGCTGTTG TGCAGACGGC TCTTCTTGGG
 601 AACGAGGAGC AGATCAAAGC CCAACTTGCA GCCGTTGAGA AGGCGAACGA AATCCGTAAT
 661 GTTGCCATTG CCGATGGTGA GATGGCGATT GCTGAGGAAC AGTATTACAT TAAAGCGCAG
 721 CTGTTGGAGC ACCTTGTGGA GCTTGTGGCC GACAAGTTTC GCATCATTGG GCAAACTGAG
 781 GATGAGAATA AGAGCTTCAG TAAGATCCAC GAGGTACAGA AGAAGTCATT TCAGGAATCT
 841 GCCTCAATCA AGGACGCGAA GCGCCGCCTT AAGCAACACT GCGAGGACGA CCTACGTAAC
 901 TTTCACGATG CCATCCAGAA AGCTGACTTG GAGGACGCCG AAGCCATGAA ACGGTTCGCC
 961 ACGCAGAAGG AGAAGTCGGA GCGGTTCATC CACGAGAACC TCGACAAACA GGACGAGGCA
1021 TGGCGTCGCA TTCAGGAACT GGAGCGCGTG TTGCAGCGCC TTGGGACGGA GCGTTTTGAA
1081 GAGGTGAAGC GCCGTATTGA GGAGAACGAC CGCGAGGAGA AGCGTAAGGT GGAGTACCAA
1141 CAGTTCCTCG ATGTATGTGG CCAGCATAAA AAGCTGCTGG AACTGTCTGT GTACAACTGC
1201 GACCTTGCGC TTCGCTGCAT GGGTATGCTG GAGGAGATCG TAGCCGAGGG CTGCAGTGCC
1261 GTCAAGTCAC GCCATGACAA GACGAACGAT GAGTTGTCTG ACCTTCGGCT GCAGGTGCAC
1321 CAGGAGTACC TGGAGGCATT CCGTCGCCTG TACAAAACTC TTGGCCAGCT TGTGTACAAG
1381 AAAGAAAAGC GCCTGGAGGA GATTGATCGC AACATCCGCA CCACACACAT TCAACTGGAG
1441 TTTGCCATTG AGACCTTTGA CCCCAACGCG AAACTACACT CCGACAAGAA GAAAGACCTA
1501 TACAAACTTC GTGCGCAGGT GGAGGAAGAG TTGGAGATGC TGAAGGACAA GATGGCGCAG
1561 GCGTTGGAGA TGTTTGGACC TACTGAGGAT GCGCTGAACC AGGCTGGTAT CGATTTTGTT
1621 CACCCTGCTG AGGAGGTTGA GTCCGGCAAC ATGGATCGCC GCAGCAAGAT GGTGGAGTAC
1681 CGTGCACACC TGGCGAAGCA GGAGGAGGTG AAGATTGCCG CGGAGCGCGA GGAGCTGAAA
1741 CGATCTAAGA TGCTCCAGAG CCAGCAG
```

Plate 29. The coding sequence of Paraflagellar rod 2 gene of *T. evansi* isolated from *Camelus dromedarius*

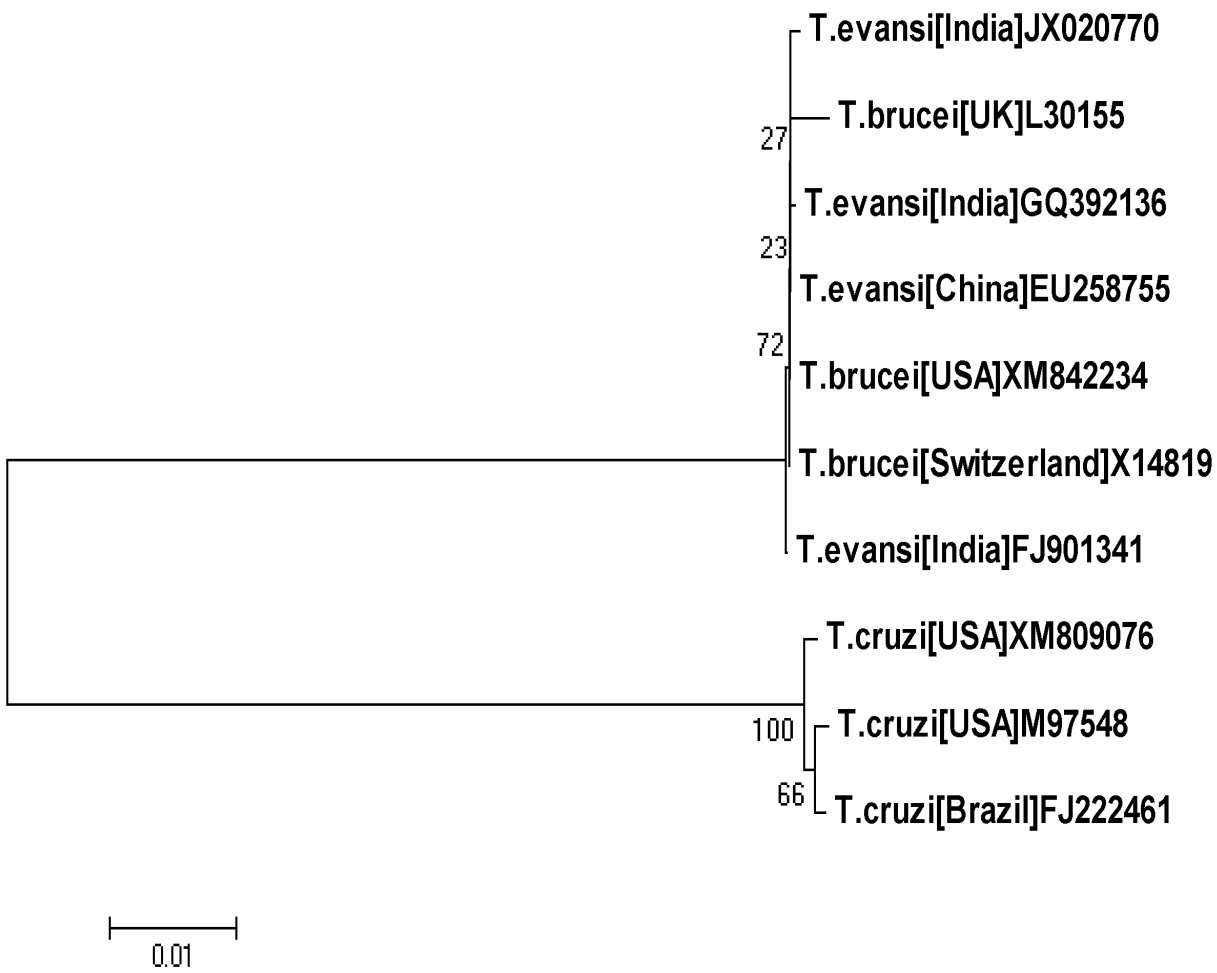

Plate 30a. Phylogenetic tree analysis of Paraflagellar rod 2 gene using the Neighbor-Joining method

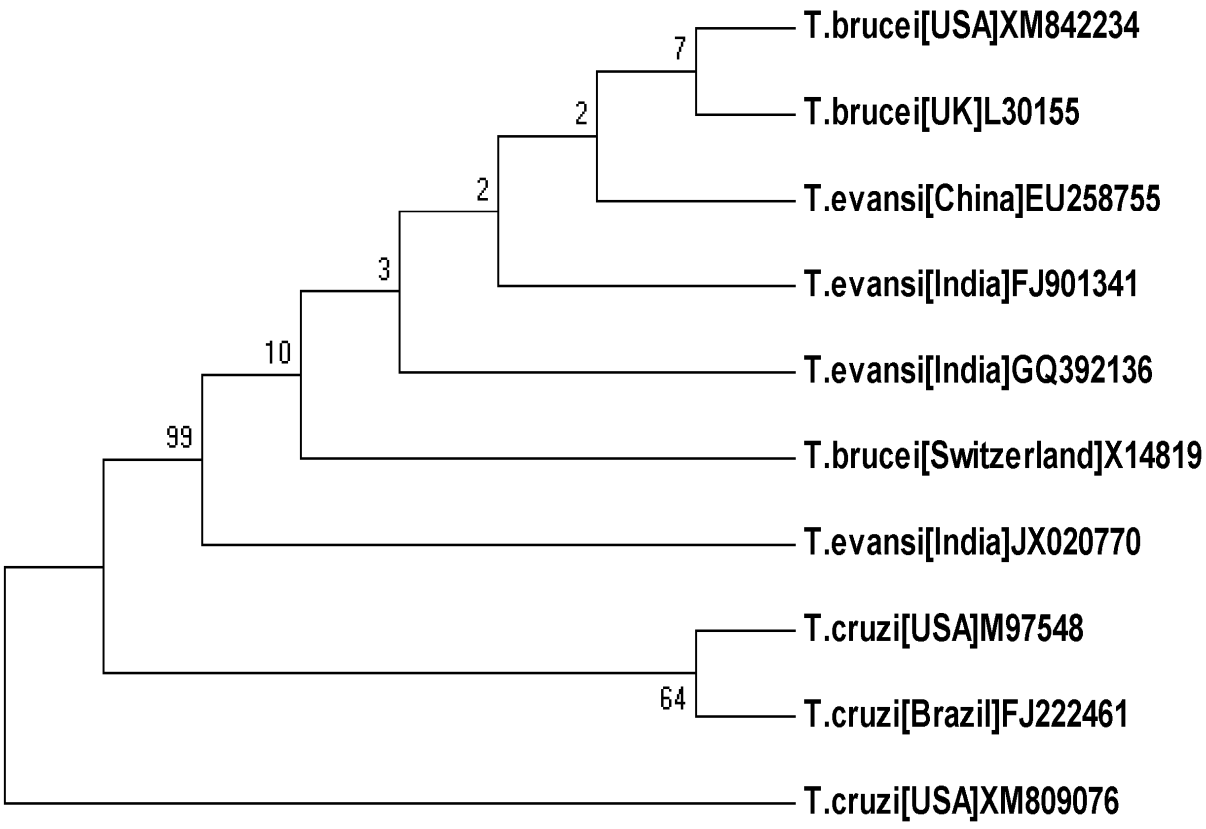

Plate 30b. Phylogenetic tree analysis of Paraflagellar rod 2 gene using maximum parsimony method

Table 4.5: Sequence identity of Paraflagellar rod 2 gene of *T. evansi* with other allied species

Identity of *pfr2* gene for *Trypanosoma evansi*	*T.evansi* [India] JX020770	*T.evansi* [India] GQ392136	*T.evansi* [China] EU258755	*T.evansi* [India] FJ901341	*T.brucei* [USA] XM842234	*T.brucei* [Switzerland] X14819	*T.cruzi* [USA] M97548	*T.cruzi* [Brazil] FJ222461	*T.brucei* [UK] L30155	*T.cruzi* [USA] XM809076
T.evansi [India] JX020770	**	99.8	99.9	99.8	99.9	99.9	83.7	83.7	99.5	83.8

T.evansi [India] GQ392136	99.8	**	99.9	99.8	99.9	99.9	83.7	83.7	99.5	83.8
T.evansi [China] EU258755	99.9	99.9	**	99.9	100	100	83.8	83.8	99.6	83.8
T.evansi [India] FJ901341	99.8	99.8	99.9	**	99.9	99.9	83.7	83.8	99.5	83.8
T.brucei [USA] XM842234	99.9	99.9	100	99.9	**	100	83.8	83.8	99.6	83.8
T.brucei [Switzerland] X14819	99.9	99.9	100	99.9	100	**	83.8	83.8	99.4	83.8
T.cruzi [USA] M97548	83.7	83.7	83.8	83.7	83.8	83.8	**	99.7	83.3	99.7
T.cruzi [Brazil] FJ222461	83.7	83.7	83.8	83.8	83.8	83.8	99.7	**	83.3	99.5
T.brucei [UK] L30155	99.5	99.5	99.6	99.5	99.6	99.4	83.3	83.3	**	83.4
T.cruzi [USA] XM809076	83.8	83.8	83.8	83.8	83.8	83.8	99.7	99.5	83.4	**

Table 4.6: Paraflagellar rod 2 gene for different species as submitted by various workers

Sl. No.	Identity of *pfr2* gene for *Trypanosoma evansi*	Accession No.	Collection Country	Reference
1.	*T.evansi*	JX020770	India	Kumar *et al.* (2012a)
2.	*T.evansi*	GQ392136	India	Ghorui *et al.* (2009)
3.	*T.evansi*	EU258755	China	Abdille *et al.* (2008c)
4.	*T.evansi*	FJ901341	India	Maharana *et al.* (2009b)
5.	*T.brucei*	XM842234	USA	Berriman *et al.* (2008)
6.	*T.brucei*	X14819	Switzerland	Schlaeppi *et al.* (2004)
7.	*T.cruzi*	M97548	USA	Beard *et al.* (1993)

8.	*T.cruzi*	FJ222461	Brazil	Silveira *et al.* (2008)
9.	*T.brucei*	L30155	UK	Gerke-Bonet and Gull (2004)
10.	*T.cruzi*	XM809076	USA	El-Sayed *et al.* (2008b)

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T.evansi_GQ392136    MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.brucei_X14819      MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.brucei_XM842234    MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.evansi_EU258755    MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.brucei_L30155      MCGKEVEGVVSPAAQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.evansi_FJ901341    MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.evansi_JX020770    MSGKEVEGVVSPADQQQPAVPEVTDITLEAARKQKIHNLKLKTACLSNEEYVQDLHVSEW 60
T.cruzi_M97548       MSYKEASGAVGPADQQQPAVPEVTDVTLEAARKQKIHNLKLKTSCLSNEEFIQDLHVSDW 60
T.cruzi_FJ222461     MSYKEASGAVGPADQQQPAVPEVTDVTLEAARKQKIHNLKLKTSCLSNEEFIQDLHVSDW 60
T.cruzi_XM809076     MSYKEASGAVGPADQQQPAVPEVTDVTLEAARKQKIHNLKLKTSCLSNEEFIQDLHVSDW 60
                     *. **..*.*.** ***********:*****************:******::******:*
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T.brucei_X14819     SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.brucei_XM842234   SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.evansi_EU258755   SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.brucei_L30155     SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.evansi_FJ901341   SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.evansi_JX020770   SETQKQKLQAAHEKAHELLASVEGGTKWSLTEAYDIKKLMRVCGLEMSVRELYKPEDKPQ 120
T.cruzi_M97548      SETQKQKLLAAHEKAQELLSSVEGGTKWNLTEAYDIKKLMRVCGLQLSVRELYKPEDKPH 120
T.cruzi_FJ222461    SETQKQKLLAAHEKAQELLSSVEGGTKWNLTEAYDIKKLMRVCGLQLSVRELYKPEDKPH 120
T.cruzi_XM809076    SETQKQKLLAAHEKAQELLSSVEGGTKWNLTEAYDIKKLMRVCGLQLSVRELYKPEDKPH 120
                    ******** ******:***:********.****************::************:

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T.brucei_X14819     FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.brucei_XM842234   FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.evansi_EU258755   FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.brucei_L30155     FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIQDELRRSQLDASEMAQVPVA 180
T.evansi_FJ901341   FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.evansi_JX020770   FMEIVALKKTMNELKQHHNKTRTVSFTGMIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.cruzi_M97548      FMEVVALKKTLNELKQHHNKTRTVSFTGTIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.cruzi_FJ222461    FMEVVALKKTLNELKQHHNKTRTVSFTGTIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
T.cruzi_XM809076    FMEVVALKKTLNELKQHHNKTRTVSFTGTIDNAIAKLEKIEDELRRSQLDASEMAQVPVA 180
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T.evansi_GQ392136   ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.brucei_X14819     ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.brucei_XM842234   ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.evansi_EU258755   ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.brucei_L30155     ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.evansi_FJ901341   ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.evansi_JX020770   ALKNIEDTMNVAVVQTALLGNEEQIKAQLAAVEKANEIRNVAIADGEMAIAEEQYYIKAQ 240
T.cruzi_M97548      VLKNLEECMNVTVVQTALLGNEEQIKAQLAAIEKAKEIRNVAIADGEMAIAEEQYYIKAQ 240
T.cruzi_FJ222461    VLKNLEECMNVTVVQTALLGTEEQIKAQLAAIEKAKEIRNVAIADGEMAIAEEQYYIKAQ 240
T.cruzi_XM809076    VLKNLEECMNVTVVQTALLGNEEQIKAQLAAIEKAKEIRNVAIADGEMAIAEEQYYIKAQ 240
                    .***:*: ***:********.**********:***:************************

T.evansi_GQ392136   LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.brucei_X14819     LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.brucei_XM842234   LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.evansi_EU258755   LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
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T.brucei_L30155      LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.evansi_FJ901341    LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.evansi_JX020770    LLEHLVELVADKFRIIGQTEDENKSFSKIHEVQKKSFQESASIKDAKRRLKQHCEDDLRN 300
T.cruzi_M97548       LLEHLVELVADKFRIIGQTEDENKPFGRIQDVQKKSFQETSAIKDAKRRLKQRCEDDLKN 300
T.cruzi_FJ222461     LLEHLVELVADKFRIIGQTEDENKPFGRIQDVQKKSFQETSAIKDAKRRLKQRCEDDLKN 300
T.cruzi_XM809076     LLEHLVELVADKFRIIGQTEDENKPFGRIQDVQKKSFQETSAIKDAKRRLKQRCEDDLKN 300
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T.evansi_EU258755    LHDAIQKADLEDAEAMKRFATQKEKSERFIHENLDKQDEAWRRIQELERVLQRLGTERFE 360
T.brucei_L30155      LHDAIQKADLEDAEAMKRFATQKEKSERFIHENLDKQDEAWRRIQELERVLQRLGTERFE 360
T.evansi_FJ901341    LHDAIQKADVEDAEAMKRFATQKEKSERFIHENLDKQDEAWRRIQELERVLQRLGTERFE 360
T.evansi_JX020770    FHDAIQKADLEDAEAMKRFATQKEKSERFIHENLDKQDEAWRRIQELERVLQRLGTERFE 360
T.cruzi_M97548       LHDAIQKADMEDAEAMKRFATQKEKSEKFIQENLDRQDEAWRRIQELERVLQRLGTERFE 360
T.cruzi_FJ222461     LHDAIQKADMEDAEAMKRFATQKEKSEKFIQENLDRQDEAWRRIQELERVLQRLGTERFE 360
T.cruzi_XM809076     LHDAIQKADMEDAEAMKRFATQKEKSEKFIQENLDRQDEAWRRIQELERVLQRLGTERFE 360
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T.evansi_GQ392136    EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
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T.brucei_XM842234    EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
T.evansi_EU258755    EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
T.brucei_L30155      EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
T.evansi_FJ901341    EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
T.evansi_JX020770    EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLALRCMGMLEEIVAEGCSA 420
T.cruzi_M97548       EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLAMRCIGMMEELVAEGCSA 420
T.cruzi_FJ222461     EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLAMRCIGMMEELVAEGCSA 420
T.cruzi_XM809076     EVKRRIEENDREEKRKVEYQQFLDVCGQHKKLLELSVYNCDLAMRCIGMMEELVAEGCSA 420
                     ******************************************:**:**:**:*******

T.evansi_GQ392136    VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.brucei_X14819      VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.brucei_XM842234    VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.evansi_EU258755    VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.brucei_L30155      VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.evansi_FJ901341    VKSRHDKTNDELSDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
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T.cruzi_M97548      IKSRHDKTNEELGDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.cruzi_FJ222461    IKSRHDKTNEELGDLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
T.cruzi_XM809076    IKSRHDKTNEELADLRLQVHQEYLEAFRRLYKTLGQLVYKKEKRLEEIDRNIRTTHIQLE 480
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T.evansi_GQ392136   FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.brucei_X14819     FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.brucei_XM842234   FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.evansi_EU258755   FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.brucei_L30155     FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.evansi_FJ901341   FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.evansi_JX020770   FAIETFDPNAKLHSDKKKDLYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIDFV 540
T.cruzi_M97548      FAIETFDPNAKKHSDAKKELYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIEFV 540
T.cruzi_FJ222461    FAIETFDPNAKKHSDAKKELYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIEFV 540
T.cruzi_XM809076    FAIETFDPNAKKHSDAKKELYKLRAQVEEELEMLKDKMAQALEMFGPTEDALNQAGIEFV 540
                    *********** *** **:***************************************:**

T.evansi_GQ392136   HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQYRGRTMPQIT- 599
T.brucei_X14819     HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQYRGRTMPQITQ 600
T.brucei_XM842234   HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQYRGRTMPQITQ 600
T.evansi_EU258755   HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQYRGRTMPQITQ 600
T.brucei_L30155     HPAEEVESGNMDRRSKMVEYRAHLAKEEEVKIAAEREELKRSKMLLSQQYRGRTMPEITQ 600
T.evansi_FJ901341   HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQYRGRTMPQITQ 600
T.evansi_JX020770   HPAEEVESGNMDRRSKMVEYRAHLAKQEEVKIAAEREELKRSKMLQSQQ----------- 589
T.cruzi_M97548      HPAEEVEDGNLTRRSKMVEYRAHLAKQEEVKIAAEREELKRSKTLQSQQYRGKTVQQITQ 600
T.cruzi_FJ222461    HPAEEVEDGNLTRRSKMVEYRAHLAKQEEVKIAAEREELKRSKTLQSQQYRGKTVQQITQ 600
T.cruzi_XM809076    HPAEEVEDGNLTRRSKMVEYRAHLAKQEEVKIAAEREELKRSKTLQSQQYRGKTVQQITQ 600
                    *******.**: **************:**************** * ***
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Plate 31. Multiple sequence alignment of Paraflagellar rod 2 Amino acid sequences with Clustal W.

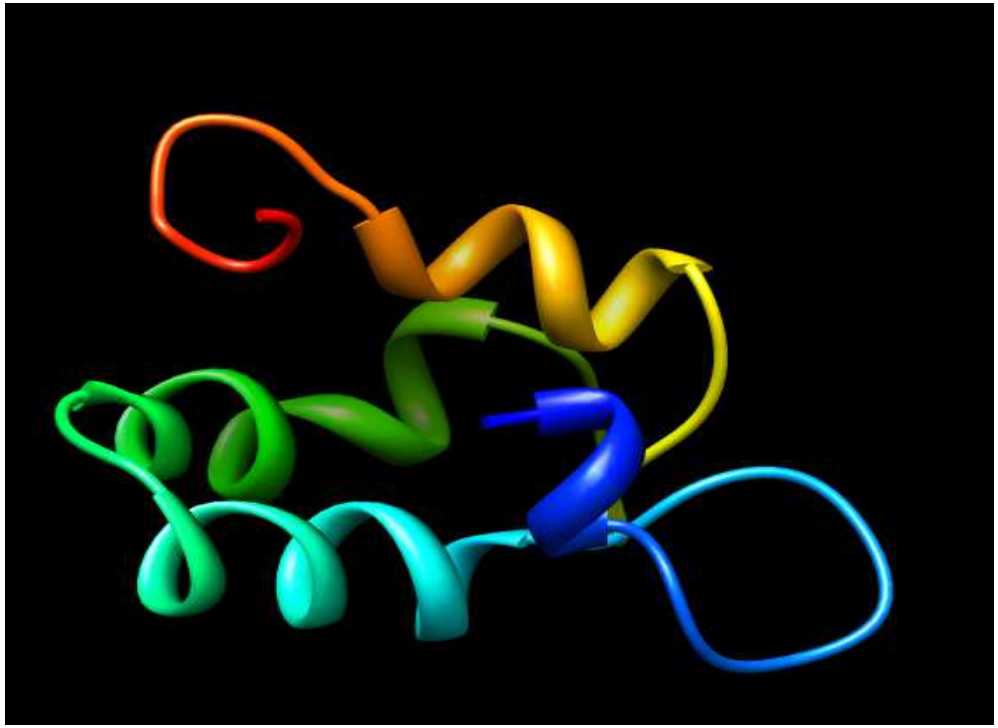

Plate 32a. Cartoon representation of 3D model of Paraflagellar rod 2 protein

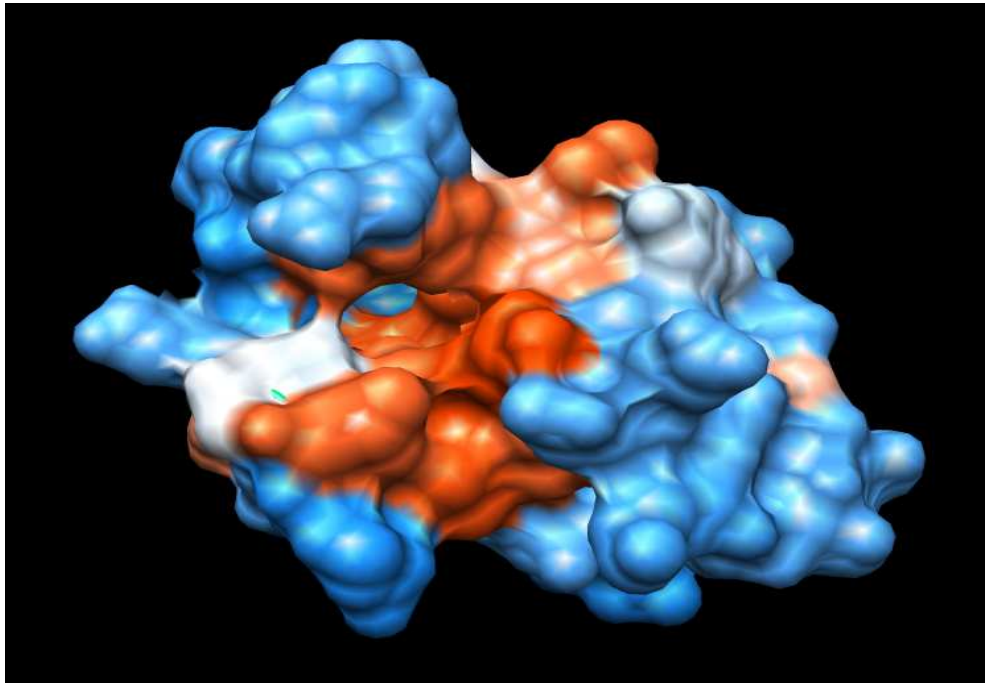

Plate 32b. Crystallographic 3D structure of Paraflagellar rod 2 protein

Various species of Trypanosomes are parasitic pathogen infecting variety of mammalian host, including human being and have great economic impact on farmers associated with animal husbandry practices. Camel trypanosomosis commonly known as ‘surra’ causes high morbidity and mortality leads to economic losses to the farmers. Chemotherapy is the main source of control of this disease but due to increasing drug resistance against *Trypanosoma evansi* the drug based control strategies has their limitation against this organism. Vaccine development against this organism is also hampered due to variation of surface proteins as antigen. Invariant proteins of *T. evansi* like Oligopeptidase B and Paraflagellar rod (1 and 2) are important virulence factor and drug target.

The reviews focused on all above mentioned subject in India or abroad, are categorized into different sub-headings-

Prevalence, diagnosis and chemotherapeutic control of trypanosomosis and development of drug resistance:

Ali and Hassan (1986) treated the healthy camels experimentally infected with *T. evansi* with isometamidium chloride (samorin) at single intravenous doses of 0.5 or 1.0 mg/kg body wt. in Sudan. After 5 to 10 min of drug administration, the camels at both dosages showed lacrimation, salivation, trembling, restlessness, frequent urination and defecation, followed by diarrhea. Moreover, the camels at the higher dose showed an unsteady gait for about an hour with hindleg weakness and walking backward. The animals fell to the ground, laid on their sides, and bent their necks into an "S" shaped curve. Three hours after the drug administration all the animals stood up and remained quiet. The treatment increased the concentration of plasma ammonia and total protein. Two hours after treatment, the activity of plasma cholinesterase was significantly reduced. The enzyme activity recovered 24 h after drug administration, but was still significantly below the control value. The treatment did not produce statistically significant changes in the hemogram of the infected camels. The results suggested that the drug should not be used

clinically against *T. evansi* infection due to its low margin of safety. If the drug is to be used at all in camels, pretreatment with an anticholinergic agent might be considered.

Lun *et al*. (1991) used the Cymelarsan in the treatment of buffaloes naturally infected with *Trypanosoma evansi* in south China. Forty buffaloes naturally infected with *T .evansi* were treated with a single dose of a new arsenical, Cymelarsan (Mel Cy) at 0.25 mg/kg to 3.0 mg/kg by intramuscular injection. All animals were cured, with the exception of two out of four animals treated with 0.25 mg/kg Mel Cy which relapsed two months after drug administration. Two out of eight buffaloes in control groups treated with a single dose of diminazene aceturate (Berenil), 3.5 mg/kg, and relapsed two months after treatment. All cured animals showed no trypanosomes in their blood when tested within one to three years after administration. A scleroma about 2 to 3.5 cm in diameter was found at the site of injection at the dose of 1.5 mg/kg Mel Cy in three of eight animals. At a dose of 3 mg/kg, Mel Cy induced obvious necrosis of the tissue at the site of injection. The results of field application proved that Mel Cy is a very active trypanocidal drug against *T. evansi*.

Kinabo (1993) reported that lack of much interest by the pharmaceutical industry to venture into development of new antitrypanosomal drugs has been a major stimulus to an intensification of research into the few existing drugs. Those indicated for animal trypanosomiasis include: isometamidium, homidium and diminazene, used primarily against *T. congolense*, *T. vivax* and *T. brucei*; and quinapyramine, mainly indicated for use against *T. evansi* infections. A great deal of research effort has focused on development of pharmacological and parasitological methodologies, which have considerably advanced our understanding on the efficacy, resistance, disposition and toxicological mechanisms of these drugs. While a clinical breakthrough has been made in the recent past, in the field of chemotherapy of *T. evansi* infections by the introduction of a new arsenic compound, melarsenoxide cysteamine, chemotherapy of *T. simiae* infections in pigs still remains a major challenge because the existing drugs are either

ineffective or too toxic for economic use. Further research into the existing drugs is a prerequisite for their optimal usage in the overall effort of improving animal health and productivity through control of trypanosomiasis.

Pathak *et al*. (1993) examined the blood samples from camels (*Camelus dromedarius*) for *Trypanosoma evansi* infection in Rajasthan. Out of 240 blood samples 18 (7.50%) were found to be infected using the wet blood Giemsa stain technique, while 76 (31.66%) camels were found to be positive for *T. evansi* antigen using the double antibody sandwich enzyme-linked immunosorbent assay (ELISA). The latter was found to be a more useful method for the detection of current infection.

Four *T. evansi* stocks with sensitivity to suramin in mice ranging from 0.05 to 160 mg kg-1 were cloned and sub-cloned by Mutugi *et al*. (1995) and sensitivity of the clones was also determined. The results suggested that it was easier to clone and sub-clone trypanosome stocks which were sensitive to suramin than those that were resistant to the action of the drug. The clones obtained from the four stocks had sensitivities to suramin which were similar to or different from the parent stocks. These results were important in view of the development of resistance for, in the presence of suramin, these resistant yet heterogeneous populations would provide the material from which selective processes could operate. These observations also suggested that the maintenance and spread of suramin-resistant trypanosomes might be curtailed by their comparative inability to establish themselves in a new host.

Talakal *et al*. (1995) studied the *invitro* and *invivo* therapeutic activity of Parthenium hysterophorus against *Trypanosoma evansi.* Crude 50% ethanolic extract of *P. hysterophorus* flowers exhibited trypanocidal activity *invitro* at all the four concentrations tested i.e. 5, 50, 500 and 1000 micrograms/ml. *Invivo* trial revealed that the extract exerted antitrypanosomal effect at doses of 100 and 300 mg/kg body wt, i.p. as evidenced by significantly reduced ($P < 0.01$) mean parasitaemia on days 3, 4, 5 and 6 when

compared with untreated control group. Further at 100 and 300 mg/kg, body wt doses, the survival period was significantly ($P < 0.05$) prolonged as compared to control group as observed by them.The extract was, however, found toxic to the animals at 1000 mg/kg dose.

Pathak *et al*. (1997) evaluated various diagnostic techniques for *Trypanosoma evansi* infections in naturally infected camels. One hundred and eight camels (Camelus dromedarius) from *Trypanosoma evansi* endemic areas of the Thar Desert of Rajasthan State, India, were evaluated by various diagnostic tests including parasitological tests (wet blood film-WBF, stained thick blood film), chemical test (mercuric chloride), biological test (mouse subinoculation-MSI), and immunodiagnostic tests based on antibody detection (double immunodiffusion test-DID, card agglutination test-CATT), antigen detection (double antibody sandwich enzyme linked immunosorbent assay-Ag-ELISA). Of the tested camels 49 were found infected using the WBF of which nine gave false negative results with the mercuric chloride test. The efficacy of MSI was 87.03 percent, while the mercuric chloride test was 60.18 percent efficient. The diagnostic efficacy of CATT (72.22 percent) was found to be much better than DID (28.70 percent). Ag-ELISA was 86.11 percent efficient in detecting trypanosomal antigens. A good correlation was found between the positive results obtained by wet blood film, CATT and Ag-ELISA. It was inferred that CATT could be used to study the seroprevalence of *T. evansi* with great ease; however, trypanosome antigen detection might give a more accurate idea of the prevalence of *T. evansi* in an endemic area.

Elamin *et al*. (1998) used the antigen detection enzyme immunoassay (AgELISA) in conjunction with parasitological examination of blood to study the enzootic situation of cameline trypanosomiasis in mid-Eastern Sudan. A one year survey showed that the infection was endemic among pastoral camels with a prevalence of 5.4% based on parasitological examination and 31.3% based on AgELISA. The infection rate was higher during the dry period (November to May) than the wet season. Young camels had a much lower infection rate as detected by parasitological techniques, but not with AgELISA. A lower prevalence of infection

was detected by buffy coat technique (BCT) in herds of camels raised by nomads compared with those kept by agropastoralists and in camels located in the southern districts of mid-Eastern Sudan.

The *invitro* effect of N,N'-diphenyl-4-R-benzamidine (where R = H, CN, Br, Cl, CH3, OCH3 and NO2) in three isolates of *Trypanosoma evansi* was studied by Gomes-Cardoso *et al*. (1999). The compounds were solubilized in dimethysulphoxide (DMSO) and tested in a concentration range of 5 to 160 micrograms/ml. The parasites were isolated from a horse, a dog and a coati. They were maintained in immunosuppressed rats, since they could not be cultured *invitro*, and further purified through a diethylaminoethanol (DEAE) column. The trypomastigotes obtained were mixed with different concentrations of the drugs and after incubation at 26 degrees C for 24 hr, the remaining parasites were counted in a Neubauer chamber. The percentage of inhibition was evaluated compared with the control, without the drugs. Most of the amidine derivatives showed high activity against the three *T. evansi* isolates, but different patterns of sensitivity to the tested compounds were observed. At least four compounds with Br, Cl, OCH3 and NO2 as substituents, were much more effective than Berenil [4,4'-(diazoamine)-dibenzamidine aceturate], the reference drug used, which was included in the same chemical class of amidines.

The drug sensitivities of 16 *T. evansi* isolates from Sudan were examined by Rayah *et al*. (1999) using two different *invitro* assays and rodent models. IC50 values (concentration which inhibited incorporation of 3H-hypoxanthine by 50%) obtained in a 40 h assay indicate that most of the isolates were resistant to suramin, a drug which has not been used in Sudan since 1975. Sensitivities for suramin in a 10-day-in vitro assay varied within a 124-fold range. The *invitro* results were confirmed by infection/treatment experiments in mice. Sensitivities *invitro* for quinapyramine varied within a 166-fold range. In mice, the least sensitive isolates were not cured with dosages up to 10 mg/kg quinapyramine. Based on *invitro* results, all isolates appeared to be susceptible to isometamidium.

Holland *et al*. (2001) examined the influence of *T. evansi* infection on the immuno-responsiveness of experimentally infected water buffaloes. In order to define the immuno-suppressive capacity of *Trypanosoma evansi* infections in buffaloes on the induction of immune responses against heterologous antigens, infected and non-infected buffaloes were vaccinated against *Pasteurella multocida* (haemorrhagic septicemia) and were simultaneously immunised with a control antigen, human serum albumin (HSA). Antibody responses against HSA were significantly reduced in *T. evansi*-infected animals, but no conclusive data were obtained on the antibody responses against *P. multocida*. Conversely, the local inflammatory response at the site of *Pasteurella* vaccination, as measured by increase in size, was significantly reduced in *T. evansi*-infected animals. These results indicated that the inductive capacity to mount humoral and cell-mediated immune responses against heterologous antigens was suppressed in *T. evansi*-infected animals. Consequently, *T. evansi* infection might interfere with the development of protective immunity upon heterologous vaccinations and could explain the poor protection of *Pasteurella*-vaccinated buffaloes in *T. evansi*-endemic areas of Vietnam.

Atarhouch *et al*. (2003) conducted an epidemiological survey of camel trypanosomosis for the first time in Morocco in 1997 and 1998. Five provinces located in the south and southeast of the High Atlas mountain chain were included in this study. A total number of 1460 serum samples were collected and tested by a card agglutination test for trypanosomosis (CATT) and enzyme-linked immunosorbant assay (Ab-ELISA) to detect anti-*T. evansi* antibodies. The overall seroprevalence was 14.1% by CATT and 18.2% by Ab-ELISA. Two very active foci were identified in Zagora and Merzouga with high Ab-ELISA prevalences of 43.3 and 35.4%, respectively, for each area. The concordance between the CATT and Ab-ELISA was 94.1%. The age-related distribution of seroprevalence showed a tendency for the infection rate to increase with age up to a maximum in the 7-10 year-old group. Clinical examination revealed that enlargement of lymph nodes was the most frequent sign in seropositive animals (79.7%). The comparison of haematocrit values of seropositive and seronegative animals showed a significant difference that indicated severe

anaemia in infected animals. Isolation of the parasite by inoculation of mice allowed then to collect fifteen *T. evansi* isolates from Zagora and three from Merzouga. The study showed that the Saharan provinces of Morocco were slightly affected by camel trypanosomosis but two hyperendemic foci, Zagora and Merzouga, were found to be sources of contamination for the surrounding regions.

Delafosse and Doutoum (2004) conducted cross-sectional studies to estimate the prevalence of *T. evansi* infection (Surra) in herds of camels from the eastern area of Chad. The risk factors associated with disease were also identified. From August 1997 to April 1998, a random sample of 2831 camels from 136 herds was selected. Blood samples were collected and examined for the presence of *T. evansi* using an antibody (card agglutination test-CATT/*T. evansi*) and a parasite detection test (buffy-coat technique-BCT). Standardized questionnaires with information about the host and management practices were collected and evaluated for their association with seroprevalence (model 1) and parasitological prevalence (model 2) as indications of host sensitivity. In both models, risk factors were selected using ordinary logistic regression (OLR) and herd effect was evaluated using a generalized estimating equations (GEE) model. The apparent prevalence was 5.3% using BCT and 30.5% with CATT. Real prevalence was estimated at 16.9% +/- 1.4 (alpha = 5%). Overall, 27.9% (BCT) and 94.9% (CATT) of the herds had a least one-positive animal. Real herd prevalence was estimated at 42.6 +/- 8.3% (alpha = 5%). Camels of the large transhumants had the highest prevalence (estimated to 30.3% +/- 2.5; 62.9 +/- 12.0 in herds). Risk factors associated with seroprevalence were age, ethnic group, length of seasonal migration and longitude of pasture area in the dry season. Risk factors associated with BCT prevalence were age, length of seasonal migration, longitude of pasture area in the dry season, latitude of pasture area in the rainy season and season of sampling.

Singh *et al.* (2004) carried a comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of natural *Trypanosoma evansi* infection in camels. A representative number of 217 camels (*Camelus dromedarius*) from different areas of western Rajasthan State, India, were examined from July 2002 to May 2003 for *Trypanosoma evansi* infection. The tests used were parasitological (wet blood film, WBF; stained thin blood smear, TBS), immunodiagnostic (double antibody sandwich enzyme linked immunosorbent assay for antigen detection, Ag-ELISA), and DNA amplification by polymerase chain reaction (PCR). These techniques were compared and the best efficiency was found with PCR. A prevalence of *T. evansi* infection was detected in 17.05, 9.67, 4.60 and 4.14% by PCR, Ag-ELISA, TBS and WBF with a sensitivity of 100, 56.75, 27.02 and 24.32%, respectively. PCR revealed a specific 227bp band in positive samples. The intensity of PCR bands was variable in different test samples depending upon the level of infection in the test samples. The history of intermittent fever, emaciation, oedema, poor body condition significantly correlated with positive serological status in ELISA as well as trypanosome DNA detection by PCR.

Gutierrez *et al*. (2006a) presented the clinical and laboratorial findings in an outbreak of abortions and high neonatal mortality attributable to *Trypanosoma evansi* infection in camels. A total of 16 females were diagnosed, 2 of which showed moderate signs of chronic form, particularly hyporexia and intolerance to exercise. The main laboratorial findings were regenerative anemia (hemolytic anemia), lymphocytic and monocytic leukocytosis, hyperproteinemia, hyperglobulinemia, hypoglycemia, serum urea increased, and serum iron decreased. The most characteristic finding in the examined females as uremia, probably due to the higher protein metabolism.

Gutierrez *et al*. (2006b) reported that trypanosomosis was a major constraint on ruminant livestock production in Africa, Asia, and South America. The principal host species affected varied geographically, but buffaloes, cattle, camels, and horses were particularly sensitive. Natural infections with *T. congolense*, *T. vivax*, *T. brucei*, and *T. evansi* have been described in goats.

Trypanosomosis in goats produces acute, subacute, chronic, or subclinical forms, being *T. vivax*, *T. congolense*, and *T. evansi*, the most invasive trypanosomes for goats. However, the role of goats in the epidemiology of trypanosomosis is largely discussed and not well understood. Thus, it has commonly been assumed that trypanosomosis presents a subclinical course and that goats do not play an important role in the epidemiology of the disease. This can partially be due to parasitemia caused by trypanosomes which has been considered low in goats. However, this assumption is currently undergoing a critical reappraisal because of goats may also serve as a reservoir of trypanosome infection for other species, including the human beings in the case of *T. brucei rhodesiense*.

Ul Hasan *et al.* (2006) carried a study on prevalence of *Trypanosoma evansi* infection in equines and camels in the Punjab region (Pakistan). A total of 170 equines and 150 dromedary camels were examined. Five (3.3%) and 6 (4%) camels were positive at parasitological and serological examination, respectively. None of the equines tested positive at any method. These results seem to indicate that *T. evansi* infection has a relatively low prevalence in the Punjab region. However, efforts must be done in order to establish control measures in affected herds and avoid dissemination of the disease.

Desquesnes *et al.* (2008) reported the first outbreak of trypanosomosis caused by *Trypanosoma evansi* in camels in France on a farm in the Aveyron Department. Five camels were imported from the Canary Islands to the farm in early July 2006, and trypanosomes were observed on a stained blood smear from one of them, which died in October. On further investigations, trypanosomes were observed in the blood of five camels, three of them indigenous to the farm and two that had been imported. On the basis of microscopical examination (morphological criteria and measurements) and serological results based on the card agglutination *T. evansi* test and PCR typing, the parasites were identified as *T*. *evansi*. After treatment with melarsomine, the infected camels rapidly became negative by parasitological tests and were negative two to four months later by serological tests.

The parasite was probably transmitted by tabanids and *Stomoxys calcitrans*, which were abundant in July to September 2006. No parasites were observed in other animals on the farm or on neighbouring farms, but some of the sheep on these farms were positive by PCR or serology.

The medicinal plant *Brucea javanica* (L.) Merr. (Simaroubaceae) is widely distributed throughout Asia where its bitter fruits have been used in traditional medicine for various ailments. Fifteen C-20 quassinoids were isolated from the fruits of *B. javanica* and examined for their in vitro antitrypanosomal activities against trypomastigotes of *Trypanosoma evansi* by Bawm *et al*. (2008). Bruceine A, bruceantinol, bruceine C, brusatol, and bruceine B showed strong antitrypanosomal activities with IC(50) values in the range of 2.9-17.8nM, which compared well with the standard trypanocidal drugs diminazene aceturate (IC(50)=8.8nM) and suramin (IC(50)=43.2nM). However, dehydrobruceine A, dehydrobruceine B, and dehydrobrusatol were about 2100, 900, and 1200 times less active, respectively, than bruceine A, bruceine B, and brusatol. The relationship of the structure and antitrypanosomal activity of these quassinoid compounds suggested that the presence of a diosphenol moiety in ring A and the nature of the C-15 side chain were important for their activities against *T. evansi*. This was the first report on the antitrypanosomal activity of isolated quassinoids.

Laha and Sasmal (2008) did the diagnosis of *T. evansi* infection in a horse stable of Eastern Region of India on the basis of examination of Giemsa stained blood smears. A high percentage (12.74%) of horses of this stable was found suffering from *T evansi* infection. This high prevalence of *T. evansi* in horses, in this area could be considered as an alarming situation which has never been explored previously in horses of Eastern Region of India. After a period of 2 months and 18 days of treatment with quinapyramine sulphate and quinapyramine chloride, reinfection with *T. evansi* in treated horses of this stable were noticed. Clinical signs of affected horses and possible causes of reinfection have been discussed by the authors.

Control measures of surra rely on diagnosis and treatment. However, with the continuous emergence of drug resistance, this tactic is failing, and the pressing need for new chemotherapeutic agents is becoming critical. Gillingwater *et al*. (2009) worked on *Invivo* investigations of selected diamidine compounds against *Trypanosoma evansi* using a mouse model. With the introduction of novel aromatic diamidines, a new category of antitrypanosomal drugs was discovered. Nevertheless, their efficacy within a *T. evansi*-infected mouse model was not known. In total, 30 compounds previously selected based on their *invitro* activity were tested in a *T. evansi* mouse model of infection. Six of the compounds were capable of curing *T. evansi*-infected mice at drug doses as low as 0.5 and 0.25 mg/kg of body weight administered for 4 consecutive days, and they were more effective than the standard drugs suramin, diminazene, and quinapyramine. After all selection criteria were applied, three diamidine compounds (DB 75, DB 867, and DB 1192) qualified as lead compounds and were considered to have the potential to act as preclinical candidates against *T. evansi* infection.

Konnai *et al*. (2009) have demonstrated that the *T. evansi* causes the disease called “Surra” in domestic animals, which is of great economic importance in South Asian countries. In order to improve the diagnosis of this disease, they endeavored to develop a real-time PCR assay for the detection and quantification of parasites in water buffaloes using specific primers for the *T. evansi* Rode Trypanozoon antigen type (RoTat) 1.2 Variable Surface Glycoprotein (VSG) gene, which is a known diverse DNA region in trypanosomes. The quantitative detection limit of the assay was 10 (2) trypanosomes per ml of blood, and the identity of the amplicon was confirmed in all assays by melting curve analysis. To evaluate the clinical applicability of this procedure, detection and estimation of parasitemia in blood samples obtained from water buffaloes and horses were conducted. *T. evansi* was detected in 17/607 (2.8%) blood samples, with parasitemia levels ranging from >10(1) to 10(7) parasites per ml of blood. Interestingly, out of the 17 PCR positive animals, 3 had previously received trypanocidal treatment and 1 had abortion history. The data indicated that

real-time PCR for the estimation of putative parasitemia levels is a quantitatively and objectively applicable technique for clinical diagnosis of Surra, and could help to understand disease stage and risk of transmission of *T. evansi*.

An outbreak of trypanosomosis caused by *Trypanosoma evansi* involving horses, camels and donkeys occurred in a farm in Israel. A longitudinal study of two outbreak phases was conducted by Berlin *et al.* (2010) which included clinical monitoring, blood smears, packed cell volume (PCV), serology and polymerase chain reaction (PCR) followed by reverse dot blot (RDB) for the molecular detection of infection. This was the first reported *T. evansi* outbreak in domestic animals in Israel. Most of the camels on the farm (8/10; 80%) were diagnosed with *T. evansi* infection whereas infection was less prevalent in the horses (3/7; 43%) and donkeys (6/13; 46%). Clinical disease was evident in 4 camels and 1 horse exhibiting characteristic clinical signs, anemia and parasitemia detected on blood smears and by positive RDB. Six other animals were diagnosed as asymptomatic latent carriers by positive RDB and 6 additional animals were only seropositive and were considered suspected carriers. A significant difference was found in the mean PCV between symptomatic and latent carriers with severe anemia observed only in the symptomatic animals. An anaphylactic-like reaction, fatal in one case, was observed in 2 camels diagnosed with severe trypanosome parasitemia immediately following treatment with melarsenoxide cysteamine. Furthermore, recurrence of infection was documented in one camel 4 months post treatment.

Gillingwater *et al*. (2010) examined *Invitro* activity and preliminary toxicity of various diamidine compounds against *Trypanosoma evansi*. Diamidines bind to the minor groove of DNA at AT-rich sites and exert their anti-trypanosomal activity by inhibiting one or more DNA dependent enzymes or by directly impeding the transcription process. In total, 67 novel diamidine compounds were tested *invitro* to determine activity against an animal pathogenic Chinese kinetoplastic *T. evansi* strain. In comparison, a human pathogenic *Trypanosoma brucei rhodesiense* strain and a P2 transporter knock out of a *Trypanosoma brucei*

brucei strain were also tested. All diamidine compounds tested in this study against *T. evansi* produced inhibitory concentration (IC(50)) values below 50 nM. The results demonstrated that these compounds were highly active against *T. evansi invitro*. In addition, preliminary *invivo* toxicity tests were performed on all 67 diamidines with 69% of the compounds showing no acute toxicity at an intra-peritoneal dose of 100mg/kg.

In a pilot study, conducted by Amer *et al.* (2011) prevalence of *Trypanosome evansi* was assessed in the blood of dromedary camels (*Camelus dromedarius*) brought to Al Bassatein abattoir, Cairo, Egypt, by mouse inoculation test out of 84 tested camels, 4 animals (4.7%) were infected. Molecular analysis was achieved by PCR amplification and sequence analysis of part of ribosomal RNA gene including 18S, ITS1, 5.8S and ITS2 regions. Despite the conserved nature of 18S region, ITS region showed obvious heterogeneity compared to analogous sequences in database. Analysis of transferrin receptor encoding gene (ESAG6) showed variable repertoire in the studied isolates, which may indicate to a novel structure of *T. evansi* population from Egypt and/or a difference in host range. Furthermore, analysis of variable surface glycoprotein RoTat 1.2 gene marker revealed some heterogeneity at this gene locus. To our knowledge, this is the first molecular analysis of *T. evansi* in Egypt.

Haridy *et al.* (2011) selected 300 camels randomly and examined them clinically and diagnosed by Giemsa stained blood smear, anti- trypanosomiasis-antibodies by ELISA and urine Thymol turbidity test for natural infection with *T. evansi* (Surra). The results showed that camels were naturally infected with *T. evansi* as indicated by stained blood film examination and/or ELISA. Infection in males was 6.0% (stained blood smears), 8.0% (ELISA) and 5.0% (urine thymol turbidity test). In females the infection rate was 9.0%, 24.0% and 12%, respectively. By correlation with suggestive clinical manifestations, ELISA proved to be more sensitive and specific (13.3%) than stained blood films (10.0%) and urine Thymol turbidity test (7.3%). Regarding humans, one out of 30 was positive as indicated by ELISA and stained blood smear but was negative by urine thymol turbidity test. The human case

was successfully treated as indicated clinically, parasitologically and serologically. This is the first reported Egyptian human case of trypanosomiasis by *T. evansi*, a neglected zoonosis.

Structure, Function and Molecular characterization of Oligopeptidase B gene:

Barrett and Rawlings (1992) reported that oligopeptidases were endopeptidases that were not proteinases in the strict sense, since they did not hydrolyse peptide bonds in proteins, but acted only on smaller polypeptides or oligopeptides. These enzymes apparently perform important, specialized biological functions that include the modification or destruction of peptide messenger molecules. Oligopeptidases have few naturally occurring inhibitors, and their distinctive specificity prevents them from interacting with alpha 2-macroglobulin, unlike the great majority of endopeptidases. The specificity of these specialized endopeptidases doubtless depends upon the three-dimensional structure of the active site, but no crystallographic structure is yet available for an oligopeptidase. Study of the primary structure of prolyl oligopeptidase has shown that it is a member of a new family of serine-type peptidases most of which are exopeptidases. The alignment of the sequences leads to the identification of some catalytic triad residues that have not yet been elucidated experimentally.

Troberg *et al*. (1996) reported that cysteine proteases are not the only parasite protease family to be possibly involved in African trypanosomiasis. Several other peptidases have now been described in *T. brucei brucei*, *T. congolense*, or *T. evansi* which are suspected of playing a role in pathogenesis. Trypanosomal serine oligopeptidases (OPs) were first described in *T. brucei brucei.* This family (composed of 80-kDa proteins) is known to catalyze the degradation of several peptide hormones, such as neurotensin or atrial natriuretic peptide. They have further been shown to retain full catalytic activity in the bloodstream of an infected host, being insensitive to plasma peptidase inhibitors.

Caler *et al.* (1998) studied the Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi* and reported that mammalian cell invasion by the intracellular protozoan parasite *Trypanosoma cruzi* is mediated by recruitment and fusion of host cell lysosomes, an unusual process that has been proposed to be dependent on the ability of parasites to trigger intracellular free calcium concentration ([Ca2F]i) transients in host cells. Deletion of the gene encoding oligopeptidase B results in a marked defect in host cell invasion and in the establishment of infections in mice. The invasion defect is associated with the inability of oligopeptidase B null mutant trypomastigotes to mobilize Ca2F from thapsigargin-sensitive stores in mammalian cells. Exogenous recombinant oligopeptidase B reconstitutes the oligopeptidase B-dependent Ca2F signaling activity in null mutant parasite extracts, demonstrating that this enzyme is responsible for the generation of a signaling agonist for mammalian cells.

During the course of studies on the peptidases of African trypanosomes, Morty *et al.* (1998) identified and purified a cytosolic oligopeptidase from the African trypanosome *Trypanosoma brucei brucei,* which called OP-Tb. The substrate specificity of OP-Tb, which preferentially cleaves peptides after pairs of basic amino acid residues, suggested that the aromatic diamidines (pentamidine and diminazene), would act as competitive inhibitors of OP-Tb. OP-Tb reversibly inhibited by the active principles of three of the five most commonly used trypanocidal drugs: pentamidine, diminazene and suramin. OP-Tb was inhibited by pentamidine in a competitive manner and by suramin in a partial, non-competitive manner.

Morty *et al.* (1999a) observed that *Trypanosoma congolense* contain a serine oligopeptidase (OP-Tb) that was released into the blood of trypanosome-infected animals. This oligopeptidase, called OP-Tc, was purified using three-phase partitioning, and ion-exchange and affinity chromatography. OP-Tc was inhibited by alkylating agents, by serine peptidase-specific inhibitors including 3,4-dichloroisocoumarin, 4-(2-aminoethyl) benzenesulfonyl-fluoride and diispropylfluoro-phosphate and by other peptidase inhibitors including leupeptin, antipain and peptidyl chloromethyl ketones. Reducing agents such as dithiothreitol enhanced activity as did

heparin, spermine and spermidine. The enzyme has trypsin-like specificity since it cleaved fluorogenic peptides that have basic amino acid residues (Arg or Lys) in the P1 position. Potential substrates without a basic residue in P1 were not hydrolysed. Although OP-Tc had weak arginine aminopeptidase activity, the enzyme clearly preferred substrates that had amino acids in the P2 and P3 positions. Overall, OP-Tc appeared to be less efficient than OP-Tb because it usually displayed lower k_{cat}/K_m values for the substrates tested. However, like OP-Tb, the best substrate for OP-Tc was Cbz-Arg-Arg-AMC (K_m= 0.72 µM, k_{cat}= 96 s^{-1}). OP-Tc preference for amino acids in the P2 position was (Gly,Lys,Arg) > Phe > Leu > Pro. The results also suggest that the P3-binding site has hydrophobic characteristics.

Morty *et al.* (1999b) reported that *Trypanosoma brucei* contained a soluble serine Oligopeptidase (OP-Tb) that was released into the host bloodstream during infection, where it had been postulated to participate in the pathogenesis of African trypanosomiasis. A single copy gene was identified encoding the *T. brucei* oligopeptidase and a homologue from the related trypanosomatid pathogen *Leishmania major.* The enzymes encoded by these genes belonged to an emerging subgroup of the prolyl oligopeptidase family of serine hydrolases, referred to as oligopeptidase B. The trypanosomatid oligopeptidases shared 70% amino acid sequence identity with oligopeptidase B from the intracellular pathogen *Trypanosoma cruzi*, which has a demonstrated role in mammalian host cell signaling and invasion. OP-Tb exhibited no activity toward the prolyl Oligopeptidase substrate *H*-Gly-Pro-7-amido-4-methylcoumarin. Instead, it had activity toward substrates of trypsin-like enzymes, particularly those that have basic amino acids in both P1 and P2 (*e.g.* benzyloxycarbonyl-Arg-Arg-7-amido-4- methylcoumarin k_{cat}/K_m = 529 s^{-1} µM $^{-1}$). The activity of OP-Tb was enhanced by reducing agents and by polyamines, suggesting that these agents may act as *invivo* regulators of OP-Tb activity. This study provides the basis of the characterization of a novel subgroup of serine oligopeptidases from kinetoplastid protozoa with potential roles in pathogenesis.

Morty *et al.* (2001) reported that a trypsin-like serine peptidase activity, levels of which correlate with blood parasitemia levels, was present in the plasma of rats acutely infected with *Trypanosoma brucei brucei*. Antibodies to a trypanosome peptidase with a trypsin-like substrate specificity (oligopeptidase B [OP-Tb]) cross-reacted with a protein in the plasma of trypanosome-infected rats on a Western blot. These antibodies also abolished 80% of the activity in the plasma of trypanosome-infected rats, suggesting that the activity might be attributable to a parasite-derived peptidase. The enzyme responsible for the bulk of this activity from parasite-free *T. b. brucei*-infected rat plasma was purified and confirmed its identity by protein sequencing. Live trypanosomes do not release OP-Tb *invitro* and propose that disrupted parasites release it into the host circulation, where it is unregulated and retains full catalytic activity and may thus play a role in the pathogenesis of African trypanosomiasis.

Bastos *et al.* (2005) reported that the 80 kDa POP Tc80 (prolyl oligopeptidase of *Trypanosoma cruzi*) was involved in the process of cell invasion, since specific inhibitors block parasite entry into nonphagocytic mammalian host cells. In contrast with other POPs, POP Tc80 was capable of hydrolysing large substrates, such as fibronectin and native collagen. Cloning of the POP Tc80 gene, whose deduced amino acid sequence shares considerable identity with other members of the POP family, mainly within its C-terminal portion that forms the catalytic domain. Southern-blot analysis indicated that POP Tc80 was present as a single copy in the genome of the parasite. Infective parasites treated with these specific POP Tc80 inhibitors attached to the surface of mammalian host cells, but were incapable of infecting them. Structural modelling of POP Tc80, based on the crystallized porcine POP, suggested that POP Tc80 was composed of an *α*/*β*-hydrolase domain containing the catalytic triad Ser^{548}–Asp^{631}–His^{667} and a seven-bladed *β*-propeller non-catalytic domain. Docking analysis suggests that triple-helical collagen access to the catalytic site of POP Tc80 occurs in the vicinity of the interface between the two domains.

Serine oligopeptidases of trypanosomatids are emerging as important virulence factors and therapeutic targets in trypanosome infections. Morty *et al.* (2005a) reported the isolation and characterization of oligopeptidase B (OpdB) and its corresponding gene from *Trypanosoma evansi*, a pathogen of significant veterinary importance. The *T. evansi* opdB gene was present as a single copy per haploid genome containing an open reading frame of 2148 bp encoding a protein of 80.664 kDa. Purified OpdB hydrolyzed substrates with basic residues in P1 (k(cat)/K(m) for carbobenzyloxy-L-arginyl-L-arginyl-7-amido-4-methylcoumarin, 337 s(-1) x microm(-1)) and exhibited potent arginyl carboxypeptidase activity (k(cat)/K(m) for Val-Lys-Arg Arg-OH, 231 s(-1) x mM(-1)). While not secreted, *T. evansi* released OpdB into the plasma of infected hosts where it retained catalytic activity. Plasma OpdB levels correlated with blood parasitemia. I*nvitro*, OpdB cleaved the peptide hormone atrial natriuretic factor (ANF) at four sites: Arg3 Arg4, Arg4 Ser5, Arg11 Ile12, and Arg27 Tyr28, thereby abrogating smooth muscle relaxant and prohypotensive properties of ANF. Circulating plasma ANF levels in *T. evansi*-infected rats were depressed from 130 to 8 pg x ml(-1), and plasma ANF levels inversely correlated with plasma OpdB activity. The *invitro* half-life of ANF in rat plasma was reduced 300-fold in plasma from *T. evansi*-infected rodents, which contains high levels of OpdB activity. Addition of OpdB inhibitors to cell-free plasma from infected rodents significantly abrogated this ANF hydrolysis. Furthermore the *invivo* ANF half-life was reduced 5-fold in *T. evansi*-infected rats. Thus, here a role for OpdB is proposed in peptide hormone dysregulation in trypanosomiasis, specifically in generating the depressed plasma levels of ANF in mammals infected with *T. evansi*.

Rea *et al.* (2006) did the expression, purification and preliminary crystallographic analysis of oligopeptidase B from *Trypanosoma brucei*. Oligopeptidase B was overexpressed in *Escherichia coli* as an N-terminally hexahistidine-tagged fusion protein, purified using metal-affinity chromatography and crystallized using the hanging-drop vapour-diffusion technique in 7% (w/v) polyethylene glycol 6000, 1 M LiCl, 0.1 M bis-tris propane pH 7.5.

Matos Guedes *et al.* (2007a) studied the molecular cloning, gene expression analysis and molecular model of Oligopeptidase B from *L. amazonensis*. A complete open reading frame of oligopeptidase B from *Leishmania amazonensis* was amplified with polymerase chain reaction with gradient annealing temperatures using primers designed for the oligopeptidase B gene from *L. major*. The 2,196-bp fragment coded for a protein of 731 amino acids with a predicted molecular mass of 83.49 kDa. The encoded protein (La_OpB) shares a 90% identity with oligopeptidases of *L. major* and *L. infantum*, 84% with *L. braziliensis*, and ~62% identity with *Trypanosoma* peptidases. The oligopeptidase B gene is expressed in all cycle stages of *L. amazonensis*. The three dimensional model of La_OpB was obtained by homology modeling based on the structure of prolyl oligopeptidases. Mapping of La_OpB model that presents a greater negative charge than prolyl oligopeptidases; results suggest a difference in the S2 subsite when compared to oligopeptidases B from Trypanosoma and bacterial oligopeptidases B. The La_OpB model serves as a starting point for its exploration as a potential target source for a rational chemotherapy.

The *Leishmania major* Genome Project identified a new oligopeptidase B that was denominated oligopeptidase B-like, herein named oligopeptidase B-2. In this study of Matos Guedes *et al*. (2008), a complete open reading frame of oligopeptidase B-2 from *Leishmania amazonensis* (PH8 strain) was amplified by PCR using primers designed for the oligopeptidase B-2 gene of *L. major*. The 2,715 bp fragment coded for a protein of 905 amino acids with a predicted molecular mass of 103,918.9 Da and theoretical pI of 5.82. The encoded protein displayed ~96% identity with *L. major* and ~75% identity with *Trypanosoma cruzi and T. brucei* oligopeptidases B-2, and ~21% identity with *Escherichia coli* and *L. amazonensis* classical oligopeptidase B. An unusual C-terminal extension was found in relation to the classical trypanosomatid oligopeptidase B. By sequence alignment, a catalytic triad (Ser 629, Asp 717 and His 758), S1 subsite (Glu 674 and Glu 676) was determined and suggested a difference in the S2 subsite of *L.*

amazonensis oligopeptidase B-2. It is also found that the oligopeptidase B-2 gene is expressed in all cycle stages of *L. amazonensis*. A phylogenetic analysis indicated that oligopeptidase B-2 is a new member of oligopeptidase B.

Cortez *et al*. (2009) analyzed sequences from genes encoding cathepsin L-like (Cat L-like) cysteine proteases from African and South American isolates of *Trypanosoma vivax* and *T. vivax*-like organisms, and evaluated their suitability as genetic markers for population structure analysis and diagnosis.

Oligopeptidase B (OPB) is a serine peptidase with dibasic substrate specificity. It is found in bacteria, plants, and trypanosomatid pathogens, where it has been identified as a virulence factor and potential drug target. In this study McLuskey *et al.* (2010) expressed active recombinant *Leishmania major* OPB and provide the first structure of an oligopeptidase B at high resolution. The crystallographic study reveals that OPB comprises two domains, a catalytic and a propeller domain, linked together by a hinge region. The structure has been determined in complex with the oligopeptide, protease-inhibitor antipain, giving detailed information on the enzyme active site and extended substrate binding pockets. It shows that Glu-621 plays a critical role in the S1 binding pocket and, along with Phe- 603, is largely responsible for the enzyme substrate specificity in P1. In the S2 binding pocket, Tyr-499 was shown to be important for substrate stability. The structure also allowed an investigation into the function of residues highlighted in other studies including Glu-623, which was predicted to be involved in the S1 binding pocket but is found forming an inter-domain hydrogen bond. Additional important salt bridges/hydrogen bonds between the two domains were observed, highlighting the significance of the domain interface in OPB. This work provides a foundation for the study of the role of OPBs as virulence factors in trypanosomatids. It could facilitate the development of specific OPB inhibitors with therapeutic potential by exploiting its unique substrate recognition properties as well as providing a model for OPBs in general.

Proteases play important roles in many biological processes of parasites, including their host interactions. In sleeping sickness, *Trypanosoma brucei* proteases released into the host bloodstream could hydrolyze host factors, such as hormones, contributing to the development of the disease's symptoms. In this study, Bastos *et al*. (2010) did the identification of the *T. brucei* prolyl oligopeptidase gene (*poptb*) and the characterization of its corresponding enzyme, POP Tb. Secondary structure predictions of POP Tb show a structural composition highly similar to other POPs. Recombinant POP Tb produced in *E. coli* was active and highly sensitive to inhibitors of *Trypanosoma cruzi* POP Tc80. These inhibitors, which prevent *T. cruzi* entry into non-phagocytic cells, arrested growth of the *T. brucei* bloodstream form in a dose-dependent manner. POP Tb hydrolyzes peptide hormones containing Pro or Ala at the P1 position at a slightly alkaline pH, and also cleaves type I collagen *invitro* and native collagen present in rat mesentery. Furthermore, POP Tb is released into the bloodstream of *T. brucei* infected mice where it remains active. These data suggest that POP Tb might contribute to the pathogenesis of sleeping sickness.

Roy *et al*. (2010) studied the clinical proteome of *Trypanosoma evansi* infection using mass spectrometry (MS) and identified over 160 proteins expressed by *T. evansi* in mice infected with camel isolate. These clinical proteomes revealed the presence of known and potential drug targets such as oligopeptidases, kinases, cysteine proteases and more.

Oligopeptidase B is a clan SC, family S9 serine peptidase found in gram positive bacteria, plants and trypanosomatids. Evidence suggests it is a virulence factor and thus therapeutic target in both *Trypanosoma cruzi* and *T. brucei*, but little is known about its function in *Leishmania*. In this study *L. major* OPB-deficient mutants (Δ*opb*) were created by Munday *et al*. (2011). These grew normally as promastigotes, had a small deficiency in their ability to undergo differentiation to metacyclic promastigotes, were significantly less able to infect and survive within macrophages *invitro*, but were virulent to mice. These data suggest that *L. major* OPB itself is not an important virulence factor, indicating functional differences between trypanosomes and *Leishmania* in their

interaction with the mammalian host. The possibility that an OPB-like enzyme (designated OPB2) in *L. major* might compensate for the loss of *OPB* in Δ*opb* was investigated via by mapping its sequence onto the 1.6 Å structure of *L. major* OPB. This suggested that the residues involved in the S1 and S2 subsites of OPB2 are identical to OPB and hence the substrate specificity would be similar. Consequently there may be redundancy between the two enzymes.

African trypanosomosis is a parasitic disease in man and animals caused by protozoan parasites of the genus *Trypanosoma*. Nagana, the cattle form of the disease, is caused by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei*. An option for developing vaccines and chemotherapeutic agents against trypanosomosis is to target pathogenic factors released by the parasite during infection, namely an "anti-disease" approach. One such pathogenic factor is oligopeptidase B (TbOPB), a trypanosome peptidase that hydrolyses Arg/Lys containing peptides smaller than 30 amino acid residues and is suspected to be involved in the hormonal deregulation associated with the disease. To better understand the role TbOPB plays in parasite physiology and host pathogenesis, oligopeptidase B null mutant parasites (Δopb) were generated in the *T. b. brucei* Lister 427 strain by Kangethe *et al.* (2012). Δopb *Trypanosoma brucei* parasites grew at a significantly faster rate *invitro*, and were as virulent as wild type strains during infection in mice. Immunohistopatholgy of infected mouse testes revealed Δopb parasites in extra vascular regions showing that TbOPB is not involved in assisting *T. brucei* parasites to cross microvascular endothelial cells. Gelatine gel analysis of Δopb null mutants showed an increase in discrete cysteine peptidase activities when compared to wild type strains. Enzymatic activity assays were carried out to identify how closely related oligopeptidases are affected by TbOPB gene deletion. A significant increase of *T. brucei* prolyl oligopeptidase (TbPOP) activity was observed, but no concomitant increase in TbPOP protein levels, suggesting that a POP-like enzyme might compensate for a loss in OPB activity in Δopb null mutants.

Oligopeptidase B, a processing enzyme of the prolyl oligopeptidase family, is considered as an important virulence factor in trypanosomiasis. *Trypanosoma cruzi* oligopeptidase B (OPBTc) is involved in host cell invasion by generating a Ca^{2+}-agonist necessary for recruitment and fusion of host lysosomes at the site of parasite attachment. The underlying mechanism remains unknown and further structural and functional characterization of OPBTc may help clarify its physiological function and lead to the development of new therapeutic molecules to treat Chagas disease. In this study Motta *et al.* (2012) conducted the size exclusion chromatography and analytical ultracentrifugation experiments which demonstrate that OPBTc is a dimer in solution, an association salt and pH-resistant and independent of intermolecular disulfide bonds. The enzyme retains its dimeric structure and is fully active up to 42°C. OPBTc is inactivated and its tertiary, but not secondary, structure is disrupted at higher temperatures, as monitored by circular dichroism and fluorescence spectroscopy. It has a highly stable secondary structure over a broad range of pH, undergoes subtle tertiary structure changes at low pH and is less stable under moderate ionic strength conditions. These results bring new insights into the structural properties of OPBTc, contributing to future studies on the rational design of OPBTc inhibitors as a promising strategy for Chagas disease chemotherapy.

Structure, Function and Molecular characterization of Paraflagellar rod (1 and 2) genes:

DeSouza and Souto-Padron (1980) reported that the paraflagellar rod, a major component of the parasite flagellum, is such a unique structure. It is a complex lattice of filaments with ultrastructural characteristics unrelated to any of the major filamentous systems of the host cells, including microfilaments, microtubules, or intermediate filaments.

Saborio *et al.* (1989) first identified the paraflagellar rod protein 2 (PFR2) gene in *T. cruzi* by pre-embedding immunoelectron microscopy with a gold-tagged secondary antibody, and they reported that it is a member of a multi- gene family of nearly 30 protozoan parasites.

Schlaeppi *et al.* (1989) working with *Trypanosoma brucei,* reported that the major protein component of the paraflagellar rod (PFR) is a single polypeptide of 600 amino acids that gives two bands of apparent molecular masses of 73 and 69 kDa in PAGE due to different conformations. They also characterized the gene coding for the PFR protein and determined that there were two identical copies in the genome.

Sherwin and Gull (1989) studied the structure of paraflagellar rod of trypanosomes through the cross-section of flagellum of *T. brucei*. The conventional axoneme is easily recognised, with its nine peripheral doublets and its central pair of microtubules, with inner and outer dynein arms, as well as radial spokes. This canonical structure is typical of a functional axoneme and is conserved in eukaryotes from protists to mammals. The lattice-like structure of the PFR is close to the axoneme and its overall diameter of 150 nm is constant throughout its length. In cross-sections, the PFR structure can be divided in three different zones called proximal, intermediate and distal, defined by their position relative to the axoneme. The individual filaments observed in all of these domains are morphologically similar but appear to be arranged differently. In the proximal and distal domains, the filaments have a diameter of 7-10 nm and intersect with an angle of 100 degrees, explaining the lattice-like aspect of the PFR. In the intermediate domain, filaments are thinner (5 nm) and intersect with an angle of 45 degrees. The proximal domain is connected to the axoneme, always in the zone between doublets 4-7, and filament connections can be seen to these specific doublets. In longitudinal sections, these connections are seen as regularly spaced fibres of 40-50 nm long in V or Y shape**.**

The previously identified major protein components of the paraflagellar rod in *Trypanosoma cruzi*, PAR 1 and PAR 2, were analyzed by Beard *et al.* (1992) to determine if they are distinct proteins or different conformations of a single polypeptide as has been suggested for other trypanosomatids. Amino acid sequence analysis showed PAR 1 and PAR 2 to be two distinct polypeptides. Antibodies specific against either PAR 1 or PAR 2 were shown to each react with a distinct band in Western blots of paraflagellar isolates of *T. cruzi* and other trypanosomatids if rigorous protease inhibition was used. The PAR 2 message was isolated and characterized by Northern blot and nucleic acid sequence analysis. Preliminary analysis of the PAR 2 gene indicates that PAR 2 is a member of a multigene family with all members residing on a single chromosome.

The PFR of trypanosomes consists of two similar proteins of approximate molecular masses of 69 and 73 kDa. Deflorin *et al.* (1994) reported the characterization of the genes coding for the 73-kDa PFR protein from *T. brucei*. Their gene product, PFR-C, is closely related to, but clearly distinct from, the 69 kDa species PFR-A. This finding indicates that the PFR fibers of the flagellum might represent heteropolymers formed by PFR-A and PFR-C, akin to the situation seen in microtubules or intermediate filaments. PFR-A and PFR-C are each coded for by a similarly organized cluster of four closely spaced, tandemly arranged genes. The 5'-untranslated region of the first gene in each cluster is different from those of the following three genes, which are identical among each other. Conversely, the 3'-untranslated regions of the first three genes of each cluster are identical, while the corresponding region of the fourth gene is different. This unusual organization leads to the generation of mRNAs which contain identical coding sequences but different 5'- and 3'-noncoding regions.

Bastin *et al*. (1996) found that the Kinetoplastida comprise a family of flagellated microbes that are defined by the presence of a network of concatenated mitochondrial DNA called the kinetoplast and a range of other unique features. One such is the

paraflagellar rod (PFR), which has an essential role in cell motility, an intricate sub-structural arrangement and an interesting phylogenetic distribution.

Miller *et al.* (1996) reported that paraflagellar rod (PFR) proteins derived from the flagellum of *T. cruzi* trypomastigotes and mixed with either Freund's adjuvant, recombinant IL-12, or adenovirus expressing IL-12, have been used to elicit cellular immune responses in mice and 100% survival against *T. cruzi* challenge infection.

Hunger-Glaser and Seebeck (1997) proved that deletion of the genes for the paraflagellar rod protein PFR-A in *Trypanosoma brucei* was probably lethal. Their results strongly indicated that the PFR structure was vital for cell proliferation in *T*. *brucei*, since deletion of the second *pfrA*/*B* locus is invariably lethal. This observation, in conjunction with the fact that the presence of a PFR structure is restricted to just a few taxonomic families (Euglenoids, Dinoflagellates and Kinetoplastids), makes it a prime target for chemotherapy with high specificity for kinetoplastid parasites.

Santrich *et al.* (1997) demonstrated a functional role for the paraflagellar rod (PFR) in motility of *Leishmania mexicana*. The PFR is a complex cytoskeletal structure running parallel to the axoneme in the flagella of kinetoplastid protozoa. The PFR is composed of a latticework of protein filaments whose major constituents are two related proteins (PFR-1 and PFR-2 in *Leishmania*). The molecular details of their assembly into PFR filaments are unknown as is the biological function of the PFR. As an approach to understanding the structure and function of the PFR in *Leishmania*, *L*. *mexicana* null mutants of *PFR*-2 was made. *PFR*-2 minus parasites grow and divide normally in culture and still express the PFR-1 protein. They lack most of the PFR structure demonstrating that the PFR-2 protein is an essential constituent of the PFR. Detailed ultrastructural analysis of the *PFR*-2 null mutant reveals the presence of a residual inner substructure of the PFR which contains PFR-1 protein, indicating that PFR-1 can polymerize in the

absence of PFR-2. The *PFR*-2 null mutant displays pronounced changes in flagellar beat waveform and forward swimming velocity, compared to wild type parasites consistent with decreased internal elastic bending resistance in PFR-lacking flagella, and indicating a functional role for the PFR in the motility of *Leishmania*.

Bastin *et al*. (1998) reported that the flagella of trypanosomatids present an important structure known as the paraflagellar rod (PFR), which was a filamentous structure that ran over the length of the flagellum along the axoneme of most of the kinetoplastid flagellates. Unlike the axoneme, which is conserved among eukaryotes, the PFR is restricted to kinetoplastids, euglenoids and dinoflagellates and is important for cell motility and for the attachment of the parasite to specific tissues in the insect vector.

Kohl and Gull (1998) reported that the flagellum of trypanosomes was attached to the cell body via a complex network of filaments (the flagellum attachment zone or FAZ) underlying both plasma and flagellar membranes which were themselves adpressed to each other. The PFR is always positioned between the axoneme and the FAZ. Some filaments connect the proximal domain of the PFR to the FAZ area. Finally, the distal domain is larger and appears to be connected to the inner face of the flagellar membrane.

Gull (1999) studied the cytoskeleton of trypanosomatid parasites and observed that trypanosomatids have many conventional structural components of flagella, they also contain an unusual fibrous body called the paraflagellar rod (PFR) that is constituted from discrete filaments, runs along the length of the flagellum, and is attached to the flagellar axoneme. The major components of the PFR are two closely related proteins have been identified in *L*. *mexicana* and *T*. *cruzi*, where they are called PFR-2, PFR-1 and PAR-2, PAR-3, respectively.

Maga and LeBowitz (1999) reported that two major protein components of the PFR have been identified in all characterized species, these will be referred to as PFR 1 and PFR 2. These proteins migrate in SDS–PAGE as a doublet of similar abundance. Depending on the organism, the mobility for PFR 1 ranges from 70,000 to 80,000 and for PFR 2 from 68,000 to 72,000. The PFR 1 and PFR 2 genes from *T. brucei*, *T. cruzi* and *Leishmania mexicana* are highly conserved across species (over 80% amino acid identity). With the exception of 20–30 residues of the N- and C-terminal sequences, sequence conservation is maintained throughout PFR 1 and PFR 2. The two sets of orthologues are also similar to each other (over 60% identity), defining a paralogous gene family that has presumably arisen from a gene duplication in an ancestral kinetoplastid.

Gadelha *et al.* (2004) studied relationships between the major kinetoplastid paraflagellar rod proteins. With the increasing availability of DNA and protein sequences, they define the levels of homology between PFR proteins and introduced a consistent nomenclature for the major PFR proteins and genes of *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania major* in order to avoid confusing or misleading annotation. They advocated a standard nomenclature for the major PFR components.

One unique structure in the flagella of trypanomastids is the paraflagellar rod (PFR). The PFR constitutes a lattice of cytoskeletal filaments that lies alongside the axoneme in the flagella. This unique and complex structure is critical for cell motility, though little is known about its molecular assembly or its role in the life-cycle of trypanosomatids. These proteins are of particular importance in *Trypanosoma cruzi*, as purified or recombinant PFR proteins have been demonstrated to be immunogenic, protecting mice from a lethal challenge with the parasite. Clark *et al.* (2005) have searched the *T. cruzi* databases and discovered two novel genes containing PFR domains. Both these genes are transcribed *invivo* and are significantly larger than the previously described PFR genes identified in *T. cruzi* (>2 Kb). Real-time PCR was used to examine the relative expression levels of six PFR genes, including the two described here, in all three stages of *T. cruzi*'s lifecycle. Database searches have further provided EST and

genomic sequence support for the presence of these genes in two other pathogenic trypanosomatids, *Trypanosoma brucei* and *Leishmania spp*. One of these genes, designated PFR5 contains a carboxy terminal SH3 domain not previously seen in PFR family genes. This proline-binding SH3 domain may play an important role in the assembly of the PFR.

Broadhead *et al.* (2006) reported that flagellar motility is required for the viability of the bloodstream trypanosome. The importance of the flagellum in many aspects of *Trypanosoma brucei* such as cellular morphogenesis, organelle positioning and pathogenicity, in addition to motility have become clear from this study.

In an attempt to identify invariant proteins with vaccine potential against African trypanosomes, Abdille *et al.* (2008a) investigated the existence of PFR1 protein in *Trypanosoma evansi* and compared its B cell epitope with that of PFR2 protein of *T. evansi* using Western blotting and immuno-precipitation assays. The PFR1 gene of *T. evansi* was amplified by RT-PCR using primers designed based on the open reading frame of PFR1 gene of *Trypanosoma brucei*. The cloned PFR1 gene of *T.evansi* was similar to PFR1 genes of *T. brucei* and *Trypanosoma cruzi*. The expressed protein from the PFR1 gene was 68.4% homologous to the PFR2 protein of *T. evansi*, and showed 99.8%, 87%, 77.9% and 77.5% homologous to the PFR1 protein of *T. brucei*, *T. cruzi*, *Leishmania mexicana* and *Leishmania major*, respectively. Western blot and immuno-precipitation assays showed that antibodies raised against PFR1 and 2 proteins in BALB/c mice recognized the PFR1 and 2 proteins, respectively, with no cross-reactivity. Immuno-agglutination assay showed trypanolytic properties of the anti-PFR1, anti-PFR2 and anti-native PFR sera. These results suggest that PFR1 and PFR2 proteins are components of native PFR antigen and do not share common B cell epitopes.

Abdille *et al.* (2008d) investigated the existence of the paraflagellar rod protein 2 (PFR2) gene in *Trypanosoma evansi* by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed based on the open reading frame of the PFR2

gene of *Trypanosoma brucei*. The PFR2 gene was cloned and the PFR2-encoded protein was expressed in bacteria. The expressed His-tag protein was purified using nickel affinity chromatography and confirmed by gel electrophoresis and Western blotting. The nucleotide sequence of the PFR2 gene of *T. evansi* showed 100% identity with the sequence of the PFR2 gene of *T. brucei* and 83.4% and 76.6% similarity with that of *Trypanosoma cruzi* and *Leishmania mexicana*, respectively. The conserved domain among various PFR2 genes present in kinetoplastids could be used as a target for the development of vaccines against multiple *Trypanosoma* species.

The eukaryotic flagellum is a highly conserved organelle serving motility, sensory, and transport functions. Although genetic, genomic, and proteomic studies have led to the identification of hundreds of flagellar and putative flagellar proteins, precisely how these proteins function individually and collectively to drive flagellum motility and other functions remains to be determined. Oberholzer *et al*. (2009) provided an overview of tools and approaches available for studying flagellum protein function in the protozoan parasite *Trypanosoma brucei*. Begin by outlining techniques for *invitro* cultivation of both *T. brucei* life cycle stages, as well as transfection protocols for the delivery of DNA constructs then describe specific assays used to assess flagellum function including flagellum preparation and quantitative motility assays. It was concluded with a description of molecular genetic approaches for manipulating gene function.

PFR is one of the major constituent proteins of the flagella of *Trypanosoma evansi* and structurally, it extends alongside of the axoneme from the flagellar pocket to the flagellum tip. This PFR is an elegant and stable lattice-like arrangement of protein filaments which is composed of two major and related proteins PFR1 and PFR2. PFR is vital for trypanosome motility and cell morphogenesis. Unlike the axoneme, which is broadly conserved among the eukaryotes, the PFR is restricted to kinetoplastids, euglenoids and dinoflagellates. So it has been considered as a vaccine target owing to its strategic location and invariable nature.

Maharana *et al.* (2011a) carried out the molecular cloning of PFR1 using pGEM-T vector and the nucleotide sequence revealed 99.8% homology and only one nucleotide change at 867bp of PFR1 ORF between the Izatnagar and China isolates. The nucleotide sequence also showed 99.8%, 82.1%, 79.9%, 72.9% homology with *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania infantum* and *Crithidia daenei*, respectively. The deduced amino acid sequence of *T. evansi* PFR1 revealed 99.7% homology between Izatnagar and China isolate. It also showed 99.8%, 92.7%, 84.7%, 82.4% homology with *T. brucei*, *T. cruzi*, *L. infantum* and *C. daenei*, respectively.

Since *Trypanosomes* can effectively evade the host immune response by displaying an array of variable surface glycoproteins, attempts for developing a protective immunogen has not been met with success. Owing to its strategic location and invariable nature PFR1 and PFR2, the two important constituent proteins of kinetoplastid flagellum, are considered as impressive candidates for vaccine development against *Trypanosomes*. Maharana *et al.* (2011b) did the Cloning and expression of paraflagellar ROD protein 2 (PFR2) gene of *Trypanosoma evansi*. PFR2 gene was cloned in pDRIVE vector. The Histidine tagged recombinant PFR2 was expressed in BL21 cells of *E. coli* using pET32a vector and subsequently purified using nickel affinity chromatography. The purity of recombinant protein was confirmed by gel electrophoresis. Further confirmation of the recombinant protein based on the immunoreactivity against the tagged Histidine residues was done by western blotting. The nucleotide sequence showed 99.9%, 82.4%, 75.3% and 74.8% sequence homology with the published sequence of *Trypanosoma brucei, T. cruzi, Leishmania infantum* and *Crithidia fasciculata*, respectively. The conserved nature of various PFR2 genes present in kinetoplastids is suggestive of its selection as a vaccine candidate against multiple *Trypanosoma* species.

Biophysical principles underlying motility of eukaryotic flagella are conserved from protists to vertebrates. However, individual cells exhibit diverse waveforms that depend on cell-specific elaborations on basic flagellum architecture. *Trypanosoma brucei* is a

uniflagellated protozoan parasite that causes African sleeping sickness. The *T. brucei* flagellum is comprised of a 9+2 axoneme and an extra-axonemal paraflagellar rod (PFR), but the three-dimensional (3D) arrangement of the underlying structural units is poorly defined. Hughes *et al.* (2012) used dual-axis electron tomography to determine the 3D architecture of the *T. brucei* flagellum. *T. brucei* axonemal repeating unit was defined and it was observed that direct connections between the PFR and axonemal dyneins, suggesting a mechanism by which mechanochemical signals might be transmitted from the PFR to axonemal dyneins. It was also found that the PFR itself was comprised of overlapping laths organized into distinct zones that were connected through twisting elements at the zonal interfaces. The overall structure has an underlying 57 nm repeating unit. Biomechanical properties inferred from PFR structure lead to propose that the PFR functions as a biomechanical spring that may store and transmit energy derived from axonemal beating. These findings provide insight into the structural foundations that underlie the distinctive flagellar waveform that is a hallmark of *T. brucei* cell motility.

6. SUMMARY AND CONCLUSION

The animal pathogenic protozoan, *Trypanosoma evansi*, leads to a wasting disease in camels, equines, cattle and other animals, commonly known as Surra. It is extensively distributed geographically with a wide range of mammalian hosts and causes great economical loss. The field control of animal trypanosomosis has, over the years, relies on two broad strategies: using chemotherapeutic agents on infected animals and vector control. In general, however, the chemotherapeutic approach is used much more widely than vector control because it is easier to kill the trypanosomes than the flies. Current methods of treatment of trypanosomes are still unsatisfactory because the number of available drugs is limited and the treatment is usually associated with severe side effects. The emergence of drug resistant trypanosomes implies failure of treatment or prevention, and if no other active drugs are available, animals have to rely on their own immune defenses alone to combat the disease. Recent effort towards the development of a vaccine against *Trypanosoma evansi* has identified several promising candidate vaccine antigens, including non-variant proteins of this parasite.

The present study entitled “Molecular characterization of Oligopeptidase B and Paraflagellar rod (1 and 2) genes of *Trypanosoma evansi* isolated from camel” was undertaken to characterize the abovementioned genes of *Trypanosoma evansi* of camel from Indian sub-continent at molecular level. This study could be helpful for understanding the pathogenesis of trypanosomosis, development of more effective drugs and evolution of vaccines.

With the aforementioned subject background, initially, the camel suffering from ‘surra’ disease in the National Research Centre on Camel, Bikaner (Rajasthan), was identified by its morphological characteristics. After confirmation of *T. evansi* infection in camel blood, 0.5 ml blood was inoculated intraperitoneally into the each experimental animal which was Swiss albino mice for propagation of parasites. The blood of mice was collected from heart region in 5 ml disposable syringe containing 0.1 ml heparin solution after dissecting the mice which had massive infection. DEAE cellulose column chromatography method was used for purification of trypanosomes. The purified trypanosomes were collected in a beaker and were pelleted by centrifugation at 1000 rpm for 10 min. The pellets were then subjected for whole genomic DNA isolation using Proteinase K digestion coupled with Phenol: Chloroform: Isoamyl alcohol extraction and subsequent ethanol precipitation. The quality of the DNA was resolved by running agarose gel electrophoresis and genomic DNA was found to be intact without much smearing. Extraction of total RNA was done by Trizol reagent as conventional method as well as using Promega SV Total RNA isolation kit following the manufacturer protocol. The extracted eluent was then subjected to agarose gel electrophoresis to confirm the presence of RNA. Two distinct bands on the plate could be visualized under UV illuminator which were confirmed as 18s RNA and 28s RNA. c-DNA synthesis and its purification was done from extracted total RNA using Clontec RT-PCR Kit. A smear like pattern was observed under UV illuminator after agarose gel electrophoresis of synthesized c-DNA.

The *opdB* gene of *T. evansi* (2092 bp) was amplified from the genomic DNA isolated from the pellets of trypanosomes using already designed forward 5’ GGACACATATGATGCAAACTGAACGTGGTCC 3’ and reverse 5’ TACGCTCATATGCTACTTCCGCAGCAGCGGCC 3’ primer sequences. *pfr1* and *pfr2* genes of *T. evansi* were amplified from both genomic DNA and c-DNA using gene specific primer sequences designed from published sequences (Accession No. EU366960 for *pfr1* and Accession No. EU258755 for *pfr2* gene). For *pfr1* gene (1769 bp) amplification, forward 5’

ATGGCCGCAGTTGACGATGCCAC 3' and reverse 5' CTATTCGAGGCGTGCCGGTG 3' primer sequences were used and for *pfr2* gene (1767 bp) amplification, forward 5' GCAGAATTCATGAGCGGAAAGGAAGTTGAA 3' and reverse 5' GACGGTACCCTGAGTGATCTGCGGCATC 3' primer sequences were used.

Polymerase chain reaction was optimized using $MgCl_2$ concentration (2.5mM) and primer annealing temperature which was 57^0C, 63^0C and 57^0C for *opdB*, *pfr1* and *pfr2* genes, respectively. Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide (0.5µg/ml) and visualized under UV light. RT-PCR of *pfr1* and *pfr2* genes showed no amplification in negative control. Subsequently, the amplicons of expected size were purified from the 1% low melting agarose gel using illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragment of interest was then ligated to the pGEM- T Easy vector as per Promega protocol with slight modification using cloning kit. Following over night incubation at 4^0C, the ligated mixture was transformed into JM109 cells by heat shock method and was plated onto LB agar containing the final concentration of ampicillin 50 µg/ml, IPTG 10 mM and X gal 20 mg/ml. The plate was incubated for overnight (16-20 hrs) at 37^0C. Both white and blue colonies were grown in the plate. Larger single white colonies were picked up individually and inoculated into LB broth containing final concentration of Ampicilin 50 µg/ml and kept in an water bath cum shaker (at 37^oC and 150 rpm) for 16 hrs. Subsequently, the plasmid DNA was isolated from all the bacterial cultures by using illustra plasmid prep mini spin kit.

Screening of recombinants (confirmation of clones) was done by Restriction Enzyme digestion of plasmid DNAs and Colony PCR of plasmid colonies. After checking the quality of the plasmid DNAs in agarose gel electrophoresis, they were subjected to restriction enzyme digestion using *Eco*RI. After 4 hrs. digestion at 37^oC in water bath, the mixture was analyzed by running agarose gel electrophoresis alongside a DNA molecular weight marker. Release of the expected size fragment confirmed the recombinants.

Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon digestion with *Eco*RI, the less intense lower band may correspond to the insert. It was found that the release of DNA fragments around 2092 bp for *opdB*, 1769 bp for *pfr1* and 1767 bp for *pfr2* gene in the 1.2% agarose gel incorporated with ethidium bromide stain.

Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers and results were analyzed by agarose gel electrophoresis using 1kb plus molecular weight marker. For *opdB* and *pfr2* gene amplifications were found in wells of white colonies but for *pfr1* gene amplification was also found in one blue colony.

After confirmation of clones of *opdB*, *pfr1* and *pfr2* genes the plasmid DNAs along with their respective forward and reverse primers were sent to Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore, for getting the sequences. The coding sequences of *opdB*, *pfr1* and *pfr2* genes according to the results obtained were of 2092 bp, 1769 bp and 1767 bp, respectively. These sequences were then matched using BLAST software. After confirmation of the *opdB*, *pfr1* and *pfr2* genes nucleotide sequences of *T. evansi* isolated from the host camel, the sequences was submitted to GenBank, NCBI database to which the assigned accession numbers are JQ909240 for *opdB*, JQ909241 for *pfr1*, and JX020770 for *pfr2* gene. Sequence analysis revealed that the length of the coding sequences of *opdB*, *pfr1* and *pfr2* genes of *T. evansi* from Bikaner, India were 2092 bp, 1769 bp and 1767 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology of *opdB*, *pfr1* and *pfr2* gene is based on the Neighbor-Joining method and maximum parsimony with100% bootstrap values. The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic tree analysis of *opdB*, *pfr1*and *pfr2* gene using maximum parsimony (MP) also showed same topology as NJ method.

Multiple sequence alignment of obtained protein sequences of *opdB*, *pfr1* and *pfr2* genes was performed with Clustal W (Clustal 2.1) at EBI.

Obtained opdB protein sequence of *T. evansi* was of 697 amino acids (GenBank Accession No. JQ909240) which showed 100% amino acid sequence identity of *T. evansi*, GenBank Accession No. AY546084, 99% of *T. brucei*, GenBank Accession No. AF078916, 98% of *T. brucei*, GenBank Accession No. XM824253, 71% of *T. cruzi* (GenBank Accession No. XM804874 and U69897), 61% of *L. amazonensis* (GenBank Accession No. EF392367), *L. donovani* (GenBank Accession No. GQ491028) and *L. infantum* (GenBank Accession No. XM001463502) and 60% of *L. major* (GenBank Accession No. AF109875).

Obtained pfr1 protein sequence of *T. evansi* was of 589 amino acids (GenBank Accession No. JQ909241) which showed 100% amino acid sequence identity of *T. evansi*, GenBank Accession No. FJ968743 and *T. brucei*, GenBank Accession No. XM838928, 99% of *T. evansi*, GenBank Accession No. EU366960, 93% of *T. brucei*, GenBank Accession No. Z25827 and *T. cruzi* (GenBank Accession No. XM804737 and AF005195), 84% of *L. infantum* (GenBank Accession No. AY702344), 85% of and *L. major* (GenBank Accession No. XM003722211) and *L. infantum* (GenBank Accession No. XM003392645).

Obtained pfr2 protein sequence of *T. evansi* was of 589 amino acids (GenBank Accession No. JQ909241) which showed 99% amino acid sequence identity of *T. evansi* (GenBank Accession No. GQ392136, EU258755 and FJ901341) and *T. brucei* (GenBank Accession No. XM842234, X14819), 98% of *T. brucei* (GenBank Accession No. L30155) and 90% of *T. cruzi* (GenBank Accession No. (GenBank Accession No. M97548, XM809076 and FJ222461).

3D structure model of obtained opdB, pfr1 and pfr2 proteins have been determined by using homology modeling protocol. Based on the maximum identity with high score and lower e-value 2XE4, 3LP5 and 2LVI were used as the template for homology

modeling. 3D models of opdB, pfr1 and pfr2 were created using Modeller9v6. Sequence identity was 52%, 33% and 32% between opdB & 2XE4, pfr1 & 3LP5 and pfr2 & 2LVI respectively. In the following steps 2XE4, 3LP5 and 2LVI were chosen as reference structure for modeling opdB, pfr1and pfr2. The final stable structure of opdB have 9 sheets, 4 beta alphabeta unit, 23 beta hairpins, 11 beta bulges, 39 strands, 22 helices, 20 helix-helix interfaces, 72 beta turns and 6 gamma turns. Similarly, the stable structure of pfr1 have 4 sheets, 1 beta-alpha-beta unit, 2 beta hairpins, 2 beta bulges, 9 strands, 35 helices, 57 helix-helix interfaces, 105 beta turns and 16 gamma turns and pfr2 have 4 helices, 4 helix-helix interact, 3 beta turns and 2 gamma turns.

The principal finding of the present study was the identification of the *opdB*, *pfr1* and *pfr2* gene in *T. evansi* from camel by sequencing the recombinant plasmid pGEM-T Easy –*opdB*/*pfr1*/*pfr2* in both directions with forward and reverse primers. The present findings therefore suggest that the identified *opdB*, *pfr1* and *pfr2* gene sequences showed a close homology with other Trypanosome gene sequences such as *T. brucei, T. evansi* and *T. cruzi*. It could therefore be suggested that vaccine with opdB, pfr1 and pfr2 proteins of trypanosomatidae parasite as the antigen could be effective against not only different strains within one trypanosome species but also against other species of the same genus.

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. Therefore, aforementioned experiments are attempted to characterize the genes of prime importance in *T. evansi* from Indian dromedaries.